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The Role of GABA in Reproductive Neuroendocrine Systems

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ABSTRACT

Gamma-amino butyric acid (GABA) is an inhibitory neurotransmitter that modulates many neuronal systems. Local GABA neurons in the hypothalamus can modulate the luteinizing hormone releasing hormone (LHRH) pulse generating system. The hormonal milieu determines the manner in which hypothalamic GABA neurons modulate the LHRH pulse generator. Using different hormonal conditions the changes in GABAergic modulation of the LHRH pulse generator may be elucidated.

Plasma estrogen levels fluctuate throughout the female reproductive cycle. In the rat medial basal hypothalamus estrogen receptive GABA secreting neurons are present. This area also contains LHRH neurons that are contacted by GABA nerve terminals. The fluctuating estrogen levels change the manner in which these systems interact.

Two animal models (intact and ovariectomized rats) were used to determine the nature of modulation of LHRH release by GABA. The first experiment determined the release of LHRH, serotonin (5-HT), and 5-hydroxyindole acetic acid (5-HIAA) during two hormonal states with each model. In the intact rat the proestrus and estrus states were examined, and in the ovariectomized rat in-vitro release was determined with ($OVXE_2$) and without (OVX) estradiol treatment. In the second experiment, one hormonal state was chosen from each model (proestrus and $OVXE_2$) to determine the effect of 0.1 mM GABA on in-vitro release of LHRH, 5-HT, and 5-HIAA. In the third experiment, $OVXE_2$ rats were used to determine the amount of GABA and muscimol (a $GABA_A$ receptor agonist) needed to cause an effect on in-vitro release of

LHRH. The fourth experiment investigated which receptor subtype was responsible for changes in in-vitro LHRH and 5-HIAA release from $OVXE_2$ rats.

These in-vitro studies were done in conjunction with several in-vivo experiments using both GABA and muscimol. These in-vivo experiments studied the effect of stimulation of GABA receptors in the median eminence on LHRH and 5-HIAA release. The results of the in-vivo studies were used to corroborate the findings of the in-vitro studies. The in-vitro experiments used a superfusion technique to study release from blocks of hypothalamic tissue. The hypothalamic tissue consisted of the medial basal and anterior hypothalamus, including the preoptic area and the suprachiasmatic nucleus. The in-vivo experiments used a push-pull perfusion technique with the cannula tip directed into the median eminence.

The intact rat model released greater amounts of LHRH than the ovariectomized rat model. The proestrus hormonal state had more LHRH released than the estrus state; however, the activity of the serotonergic system was greater during estrus. The LHRH release in the ovariectomized rat model was similar during both hormonal states but serotonergic activity was greater in the $OVXE_2$ rat. GABA stimulation did not change LHRH release although it did change serotonergic activity, during the proestrus hormonal state. In the $OVXE_2$ rat GABA stimulation decreased LHRH release, while serotonergic activity was not altered.

The minimum amount of GABA necessary to cause an effect was 0.01 mM; however, the 0.1 mM dose had an equal effect. The only difference found with the $GABA_A$ receptor agonist muscimol was a decrease between the 10^{-7}M dose and the 10^{-5}M dose. The data from the receptor subtype experiment was inconclusive. None of the receptor subtype specific agonists and antagonists significantly increased or decreased LHRH and 5-HIAA release. The in-vivo experiments showed that GABA could decrease LHRH release from the median eminence, while muscimol could change 5-HIAA release.

The modulation of the LHRH pulse generator by GABA is dependent on the hormonal milieu created by the ovary. During proestrus, when estradiol levels begin rapidly rising, LHRH release is unaffected by GABA. However, in the $OVXE_2$ rat, when estradiol is at a low constant level, LHRH release is decreased by 0.1 mM GABA. The LHRH release decrease in $OVXE_2$ rats also could be caused by 0.01 mM GABA. This GABA mediated inhibition of LHRH release is also seen in-vivo. The interaction of GABA and serotonin systems within the hypothalamus is dependent on the hormonal state. Serotonergic activity during proestrus, but not in $OVXE_2$ rats, is changed by GABA treatment. In-vivo experiments on $OVXE_2$ rats show an interaction between $GABA_A$ receptors and serotonin metabolism. The interactions between GABA and serotonin within the hypothalamus, and how they affect LHRH release from the hypothalamus, is dependent on the cyclic release of ovarian hormones.

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DEDICATION

You always said, "People don't do what they believe in, they just do what's most convenient, and then repent." I always said, "Hang on to me baby and let's hope the roof stays on." -Bob Dylan & Sam Shepard

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INTRODUCTION

Reproductive neuroendocrine system:

Reproductive functions in mammals are governed by a system that resides within the hypothalamus, pituitary, and gonads. This hypothalamic-pituitary-gonadal system is very complex and interdependent, with multiple feedback mechanisms between the tissues in this system. The proper functioning of any portion of the system is accomplished through hormones and neurotransmitters which mediate both positive and negative feedback mechanisms controlling ovulation.

The reproductive neuroendocrine system remains quiescent prior to puberty, whereupon it acquires the cyclic hormonal characteristics of the female or the more tonic, negative feedback system of the male. The different gonads utilize different feedback strategies to regulate the release of luteinizing hormone (LH). In males a tonic feedback of testosterone from the testes on the pituitary maintains reproductive competency. However, in females the ovarian estradiol (E_2) feedback reflects the E_2 from the dominant follicle and ovulation of this oocyte occurs after the spiking LH level in the plasma acts on the follicle.

Reproductive effectiveness (fecundity) depends on the E_2 and progesterone (P_4) conditioning of receptivity of the uterus for a very brief part of the ovarian cycle. The proper preparation of the uterus for implantation of the blastocyst is essential for reproduction. The uterine endometrium must be hormonally prepared and the release of the oocyte properly timed so that ovulation, fertilization, and implantation occur within a narrow time frame. If the oocyte is not present at this time, any fertilization that occurs can not result in successful implantation and subsequent development of the fetus. The process of releasing an oocyte is known as ovulation. The timing of ovulation is critical to the success of reproduction. An oocyte must be released when the subsequent fertilization will result in a successful pregnancy. The appropriate neural signal for ovulation to occur is conditioned and coordinated by the follicle

destined to ovulate. The signal is not completely understood, and it is created by the interactions of several neurotransmitter systems. This study will try to determine how some of these neurotransmitters interact to influence the neural signal.

The neural signal determining the onset of ovulation arises in the hypothalamus. The portal circulation carries this signal from the median eminence of the hypothalamus to the anterior pituitary. Once the pulsatile signal reaches the anterior pituitary, gonadotropes are stimulated to release pulses of LH. The cyclic release of LH by the hypothalmic-pituitary unit is coordinated by the stimulatory and progressively increasing feedback signal provided by the ovarian secretion of estradiol. A surge of LH pulses is the trigger for ovulation. The buildup of LH release capacity during the follicular phase of the cycle provides the proper massive release of LH necessary for the acute release of oocytes at the midcycle surge. The chemical messenger which causes the gonadotrope to release LH is a decapeptide known as luteinizing hormone releasing hormone (LHRH).

LHRH release is driven by a pulse generator located in the hypothalamus. Both the amplitude and frequency of LHRH pulses have a profound effect on LH secretion from the pituitary. Increasing both the amplitude and frequency of LHRH pulses will increase the mean LH levels as well as increase the amplitude of LH pulses. However, if the frequency is greater than three pulses per hour, the amplitude of LH pulses decreases. An interesting phenomenon is the self priming effect of LHRH on LH release. Apparently small amounts of LHRH increase the sensitivity of LH gonadotrophs to subsequent pulses of LHRH, thereby enhancing the effect of LHRH. This is then followed by a desensitization of the gonadotrope to LHRH (35). In rats the frequency of LHRH pulses remains constant (about one pulse every fifty minutes) throughout the estrous cycle. However, a significant increase in amplitude of LHRH pulses occurs during proestrus (63). In freely behaving primates, the luteal phase of the cycle is characterized by a decrease in the frequency of the LHRH signal. If this

decrease is prevented by infusion of LHRH, the next four follicular phases (from the first day of menstruation until the first day of sustained high serum progesterone) are lengthened while the length of the luteal phase remains unchanged (42).

Effect of estradiol on the pituitary:

The ovarian steroid hormone estradiol can affect the release of LH from gonadotropes. Immunoprecipitation techniques in rats show LHRH can stimulate the synthesis of LH by gonadotropes, and this action is potentiated by estradiol (70,65). LH released by anterior pituitary tissue removed from rats during proestrus shows a sustained sensitization to LHRH, while tissue removed during diestrus shows sensitization followed by desensitization (58). Estradiol lowers the EC_{50} (the concentration needed to half maximally stimulate gonadotropes) of LHRH on cultured female rat anterior pituitary cells (36). Estradiol can also almost completely block the ability of pituitary cells to metabolize LHRH (15). During times of high circulating estradiol levels the metabolism of LHRH by gonadotropes is greatly reduced. This, coupled with the potentiation of LH synthesis and increased LH secretion for each pulse of LHRH caused by estradiol, shows that estradiol can considerably amplify the hypothalamic signal by its action on the pituitary.

Relationship of LHRH pulse generator and LH:

The pulses of LHRH released from the median eminence correlate with LH pulses released from the anterior pituitary. The LHRH pulse generator is able to synchronize the release of LHRH from many neurons simultaneously. The anatomical location of this pulse generator may be within the intrinsic network of hypothalamic LHRH neurons (18). All major LH secretory episodes are immediately preceded by a transitory increase in the concentration of LHRH in the portal circulation (47). Electrophysiological evidence from primates also supports hypothalamic control of LH

release. Neuronal multiunit activity in the medial basal hypothalamus (MBH) of rhesus monkeys is abruptly increased during LH pulses. This activity is characterized by a sudden and major increase in the frequency of action potentials (40). The ability of this pulse generator to synchronize LHRH release into the portal circulation is critical for the subsequent release of LH into the periphial circulation. This synchronicity of the LHRH pulse generator is a poorly understood phenomena. These experiments will try to clarify some of the influences on the release of LHRH pulses from the median eminence.

Modulation of the LHRH pulse generator:

The LHRH pulse generator can undergo considerable modulation by several neurotransmitter systems. Noradrenergic pathways originating in the locus coeruleus, serotonergic pathways from the raphe nucleus, as well as hypothalamic opiate and GABAergic neurons have all been implicated as modulators of the LHRH pulse generator. The interactions of these systems with LHRH neurons can change many aspects of the LHRH pulse generator.

Noradrenergic systems exert a stimulatory effect on LHRH release. Norepinephrine (NE) fibers have axon terminals on LHRH cell bodies within the medial preoptic area of rats (80). Intracerebroventricular norepinephrine injection in rats amplifies and prolongs the release of LH after electrical stimulation of the medial preoptic area (MPO). Coincidentally, the GABA A and B receptor antagonists, bicuculine and phaclofen, can increase this response to norepinephrine (NE) (29).

In-vivo studies in primates demonstrate that norepinephrine transmission normally facilitates pulsatile LH output by acting through an alpha receptor mechanism (66). In-vivo push-pull perfusion (PPP) experiments in rhesus monkeys reveal that norepinephrine release is pulsatile and synchronous with LHRH and LH release. Infusion of norepinephrine or methoxamine (an $alpha_1$ adrenergic agonist) stimulates

LHRH release, while intravenous injection of prazosin (an alpha$_1$ adrenergic blocker) suppresses LHRH release (72). In the rabbit PPP shows an increase in the mating induced release of LHRH and norepinephrine from the medial basal hypothalamus, as well as an increase in release of LHRH from the anterior hypothalamus (38). The stimulatory effect of norepinephrine on the LHRH pulse generator is evident in several species.

Serotonin can have a paradoxical effect on LHRH neurons. Serotonin acts at the level of the median eminence in rats as a facilitatory neurotransmitter involved in preovulatory LHRH release (76). Clinical studies have shown an inhibitory effect of hypothalamic serotonin receptors. These studies showed that the inhibitory opioid control of LH release is mediated by serotonergic receptors in males (21). Estradiol may be the hormonal trigger for changing serotonin's effect from inhibition to facilitation of LHRH release. Serotonin will significantly decrease the frequency of LHRH release in an OVX rat, and significantly increase the frequency in an OVXE$_2$ rat (54). The effects of ovarian steroids are anatomically specific. Central serotonergic neurons, innervating the MPO, can stimulate the surge of LH released when OVXE$_2$ rats are treated with progesterone (32). Estradiol and progesterone induce an increase in the synthesis of serotonin in the MPO, but not in the MBH of OVX rats (39).

The cyclic nature of serotonergic activity in the hypothalamus is important for LH release. A properly timed serotonin circadian rhythm is facilitatory to LH surges. Progesterone will amplify this rhythmic release of serotonin. The intensity of the serotonergic signal may relate to the size of the daily fluctuations in hypothalamic serotonin content (79). The facilitatory effect of serotonin on LH secretion is lost in OVX rats chronically exposed to estradiol. Pharmacological amplification of signals in serotonergic circuits by 5-hydroxytryptophan will reinstate the facilitatory effect of estradiol (78). The ovarian steroids estrogen and progesterone affect the circadian rhythm of serotonin which modifies the release of LHRH from the hypothalamus.

Behavioral influences of ovarian steroids may be mediated by serotonin. The stimulatory effect of progesterone on sexual behavior may occur via a serotonergic mechanism involving 5HT-2 receptors (81). Tonic inhibition of sexual behavior is related to serotonin levels in the ventromedial nucleus (VMN). Facilitation of sexual behavior is greatest when depletion of serotonin in the VMN is maximal. As serotonin levels rise in the VMN, the facilitation of sexual behavior decreases (23). Serotonin has a predominantly inhibitory action on the electrical activity of VMN neurons. Estradiol causes a higher proportion of VMN neurons to respond to serotonin (41).

Opioid neurotransmitter systems can inhibit the release of LHRH into the portal circulation. Naloxone (an opiate antagonist) increases both the frequency and amplitude of pulsatile LH release in both sexes, but opioids have no effect on the LH response to LHRH (31). A sustained decrease in the inhibitory opioid tone, on the morning of proestrus, in naloxone treated rats advances the onset of the LH surge (5). Intravenous infusion of naloxone (two mg/hr) at 11:00 am on proestrus induces a surge like release of LH resembling that normally seen in the afternoon (4). The effects of opiates can be decreased by ovarian hormones. Either intravenous or central administration of naloxone readily stimulates hypothalamic LHRH and pituitary LH release in ovariectomized rabbits. However, no significant stimulation is seen in intact rabbits (62). In male rats baclofen, a GABA-B agonist, can completely suppress the naloxone stimulated release of LH (51). GABAergic transmission can also increase the stimulatory response to norepinephrine in female rats (14). The role of GABA in modulating the LHRH pulse generator is not well defined. This study will help to define this role, and also determine the relationship between GABA sytems and serotonin systems in modulation of the LHRH pulse generator.

Stimulatory effects of GABA are seen during tonic gonadotropin secretory conditions, whereas inhibitory effects can be seen in phasic secretory conditions (50). There may be opposite actions of GABA on LHRH release depending on the site of

action within the hypothalamus (53). In the MPO and the MBH, GABA-A and GABA-B receptor stimulation can decrease the concentration of NE and the NE turnover rate in ovariectomized estrogen primed (OVX-E_2) rats (2). GABA-A and GABA-B receptor blockade can potentiate the stimulation of LHRH neurons resulting from stimulation of NE transmission (29). GABA fibers are present throughout the hypothalamus, and many GABA cell bodies are present within several nuclei of the hypothalamus (75). The MPO contains GABAergic neurons in direct synaptic contact with LHRH neurons (44). Direct synaptic connections between glutamic acid decarboxylase (GAD) and LHRH containing neurons, and catecholaminergic neurons are seen in the MPO. Some of these connections are from brainstem NE neurons, while the majority may represent local dopamine containing cells (45).

The action of GABA-A receptor stimulation on NE turnover rate is highly specific. It occurs only in the MPO and is not seen in other hypothalamic nuclei, the nucleus accumbens, or the mediocortical amygdala (25). At the time of increasing plasma LH levels, MPO GABA release rates decrease, while NE release rates increase in OVX-E_2 rats. The negative feedback effect of estrogen on LH is manifested in higher release rates of GABA and lower release of NE in the MPO of rats (16). However, during the negative feedback effect of estrogen on LH in rhesus monkeys, GABA release rates from the MBH decreased (24). Opiate receptor blockade in male rats causes a stimulation of LH release. This stimulation can be blocked by administration of baclofen. GABA-B receptor stimulation may be responsible for the tonic inhibitory effect of endogenous opiate peptides on LH secretion in male rats (51). Also in male rats stimulation of GABA-A receptors on LHRH neuron cell bodies can increase LHRH release from hypothalamic slices. When firing of the axon is blocked by tetrodotoxin, GABA exerts an inhibitory effect upon LHRH release, by acting directly on LHRH nerve terminals (59).

Gamma-aminobutyric acid (GABA):

GABA is a ubiquitous inhibitory neurotransmitter that is active at approximately 20-40% of brain synapses. GABA may have a secondary role in fine-tuning the actions of other neurotransmitters without being crucial for a resulting signal transmission (52). The interaction of GABA with other neurotransmitter systems is the basis for much of the modulation that occurs in the central nervous system.

The production of GABA is accomplished by using Kreb's cycle intermediates to generate glutamate. The glutamate is compartmentalized depending on whether it is destined to become glutamine or GABA (12). The Kreb's cycle intermediates are derived from a pathway involving pyruvate carboxylase (11). An alternate pathway exists, originating with the fatty acid putrescine, which doesn't require glutamate but rather uses gamma-aminobutyraldehyde (1).

GABA is also recycled from the synaptic cleft. The human brain GABA transporter has recently been cloned and sequenced (57). A sodium and chloride dependent high affinity GABA transport system exists in rat brain plasma membranes (37). Reuptake of GABA is driven by components of the proton electrochemical gradient, the pH difference, and the membrane potential. It is electrogenic and occurs in exchange for protons (30). The levels of GABA within a postsynaptic cell can regulate the functioning of the GABA-A receptor (84).

The GABA receptor exists in two types known as A and B. The effects produced by GABAergic neurotransmission depend on the type of receptor with which GABA interacts. There are also two functional components of synaptasomal GABA release, a phasic one, and a tonic one. The phasic component is normally initiated by a voltage sensitive Ca^{2+} channel that is functionally and pharmacologically distinct from any previously described. Calcium dependent GABA release depends on Ca^{2+} entry into the neuron terminal and not depolarization (74).

More is known about the GABA-A receptor. Muscimol, a well characterized agonist, exists for the GABA-A receptor, as does bicuculine, a specific antagonist. The receptor is associated with the benzodiazepine receptor, the barbiturate receptor, and a chloride ion channel. Many ligands are available that bind to these sites. The receptor constitutes a gated chloride channel; the allosteric modulation of which has received considerable study. Expressing the alpha-1, beta-1, and gamma-2 subunits from rat brain in sea urchin eggs results in a receptor which is modulated in a qualitatively similar manner as the in-vivo receptor (49). Expression of alpha-1 and beta-1 subunits is sufficient to form GABA-A receptors that contain binding sites for GABA, barbiturates, and also benzodiazepine receptor ligands (48).

Benzodiazepines potentiate the activity of the GABA gated chloride channel intrinsic to GABA-A receptors. When the three different alpha subunit variants are expressed with beta-1 and gamma-2 subunits in mammalian cells, heterogeneity in GABA-mediated potentiation of benzodiazepine binding is seen. These different receptors reveal the structural basis for benzodiazepine receptor heterogeneity (64). A specific steroid recognition site is part of the expressed GABA-A receptor chloride channel complex. This site is functionally coupled to the benzodiazepine and GABA-A receptors (43). Allosteric modulation within the GABA-A receptor is similar in both trout and rat brain membrane, indicating a conservation of this type of GABA receptor over vertebrate evolution (19).

Considerable differences in the distribution of GABA receptors exist between males and females. Several hypothalamic nuclei important for sexual differentiation show different numbers of GABA-A receptors. The number of GABA-A receptors in the medial basal hypothalamus and the preoptic area of male rats is five times higher than in diestrous female rats (33). In hormonally sensitive nuclei estradiol decreased binding to GABA-A receptors while a subsequent dose of progesterone returned binding to control levels in ovariectomized and adrenalectomized rats (69). The

fluctuating levels of ovarian steroid hormones during the cycle change the level of GABAergic neurotransmission in these nuclei. In male rats there are between two and two and a half times more GABA-A than GABA-B receptors in these same hormone sensitive nuclei (13). Estradiol treatment of ovariectomized rats down regulates GABA-B receptors in the suprachiasmatic nucleus (22).

The properties of the GABA-B receptor are very different from the properties of the GABA-A receptor. The GABA-B receptor only has one recognition site, a binding site for GABA. It is not associated with a Cl^- channel but appears to be coupled to Ca^{2+} and K^+ channels. Activating these receptors decreases the amplitude of Ca^{2+} currents and increases K^+ conductance. GABA-B receptor binding is inhibited by guanyl nucleotides (22). Pharmacologically distinct subsets of GABA-B receptors are distinguished by their ability to affect second messenger systems (68). GABA autoreceptors regulating the release of both newly taken up and endogenous GABA are present in the human brain and appear to belong to the GABA-B subtype (9). GABA-B autoreceptors are present in the median eminence of the rat hypothalamus (6). GABA-B presynaptic receptors can be stimulated by baclofen on noradrenergic, dopaminergic, serotonergic, and glutaminergic nerve terminals in the central nervous system (77).

Although a good agonist, baclofen, exists for the GABA-B receptor, no well accepted antagonist exists. The best characterized antagonist, phaclofen, requires a fairly high concentration (10^{-4}M) to be effective (17). GTP binding proteins (G-proteins) couple GABA-B receptors and different effector systems. These G-proteins are sensitive to pertussis toxin (PT). PT treatment will block the ability of baclofen or depolarization to affect Ca^{2+} activated K^+ channels in synaptosomes (73). The GABA-B receptor mediated potentiation, of the beta-adrenergic receptor stimulation of adenylate cyclase, is attenuated by PT treatment (83). PT treatment blocks postsynaptic hyperpolarization (mediated by K^+ channels) but not presynaptic

inhibition (mediated by Ca^{2+} channels) in the rat dorsal raphe nucleus (14). Some G-proteins that link GABA-B receptors with ion channels are not sensitive to PT.

GABA and serotonin interactions:

GABAergic and serotonergic systems interact in several different areas of the central nervous system. Depending on the area of the central nervous in which they occur, these interactions can mediate a variety of specific functions. These functions are as diverse as head twitch behavior and release of hypothalamic hormones. The anatomical relationships between these systems determine the type of function mediated by their interactions.

In hippocampal pyramidal cells, a PT sensitive G-protein links both the GABA-B receptor and the serotonin 1a (5HT-1a) receptor to the K^+ channel. This G-protein may directly couple these receptors to this channel; activators of the G-protein mimic the effects of baclofen and serotonin and appear to block their actions (48). The same system of receptors (GABA-B and 5HT-1a), effector (G-protein), and ion channel (K^+) exists in dorsal raphe serotonergic neurons.(49) The potassium conductance stimulated by the GABA-B and 5HT-1a receptors in these neurons is identical in amplitude and voltage dependence (50). In frontal cortex, GABA-B receptors inhibit the release of serotonin. This causes the up regulation of another serotonin receptor (5HT-2). This change is reflected in characteristic behavioral changes (27). In mouse cortex, GABA-B receptors modulate 5HT-2 receptor function at the level of signal transduction, causing an inhibition of inositol phosphate formation (26).

Striatal serotonin release is increased by a blockade of GABA-A receptors. This blockade will potentiate the effect of stimulation of the lateral habenula-dorsal raphe pathway which connects the dorsal raphe with the striatum (34). In the cat caudate nucleus, the paradoxical inhibitory influence of glutamate on serotonin release is mediated via stimulation of GABA-A receptors. GABAergic interneurons are

activated by stimulation of N-methoxy-D-aspartate (NMDA) receptors by glutamate causing an inhibition of serotonin release (8). In cerebellar Purkinje cells serotonin, applied at currents that produced minimal effects on the spontaneous firing rates of Purkinje cells, modifies GABA effects in a biphasic manner. Serotonin initially decreased GABA mediated inhibitition, and secondarily, serotonin either continued to inhibit, or most frequently elicited an augmentation of the GABA response. The effects of serotonin on GABA inhibition seem to be governed by an intrinsic property of the Purkinje cell, which may relate to the firing rate of the cell (71).

In the ventral medulla oblongata, the colocalization of GABA and serotonin within the same cell bodies is limited to the nucleus raphe magnus/nucleus paragigantocellularis (55). The raphe nuclei are the anatomical sites for the inhibitory GABA influence on cerebral serotonergic neurons (56). GABAergic interneurons, located in the dorsal raphe, inhibit serotonin release and synthesis in distant axon terminals, as well as locally in dendrites and cell bodies of serotonergic neurons (67). A serotonergic pathway activated by the medial raphe nucleus inhibits the proestrus surge of LH. This inhibition is mediated by GABAergic neurons (61). About 5% of the synaptic contacts on LHRH neuron dendrites are from serotonergic neurons. Glutamic acid decarboxylase (GAD) containing axons from local neurons make synaptic contact with LHRH neurons in the medial preoptic area (28).

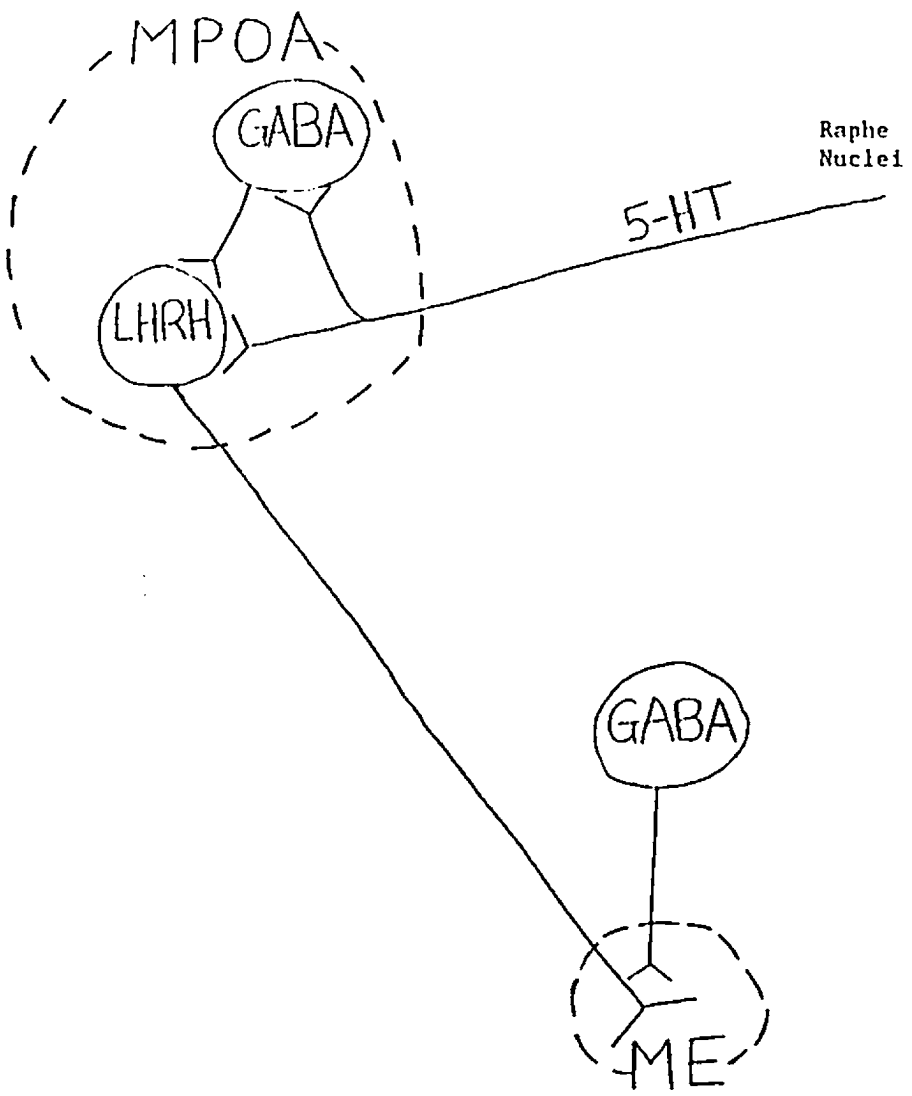
Activation of the serotonergic system, by systemic administration of 5-hydroxytryptophan, brings about a transient activation of the GABAergic system within the medial basal hypothalamus. Both the synthesis and release of GABA are affected by activating serotonin neurons (3). In the suprachiasmatic nucleus (SCN), about 41% of the serotonergic nerve terminals are directly apposed to at least one GABA nerve terminal. Electron microscopy shows unmyelinated axonal varicosities labeled with [^{3}H]5-HT facing similar structures containing immunocytochemically labeled glutamic acid decarboxylase (GAD). The serotonergic and GABAergic inputs to the SCN

belong to distinct afferent systems (10). Arcuate nucleus implants of GABA and serotonin affect serum LH and prolactin levels respectively. Serotonin significantly increased prolactin, while GABA significantly decreased LH levels. GABA decreased LH pulse frequency but not amplitude (60). The extent of the interactions between local GABA neurons and serotonergic axons projecting from the raphe nuclei is not known. These exepriments helped to determine the extent of these interactions.

Summary of goals:

GABA and serotonin influence plasma LH levels through hypothalamic interactions that alter the release of LHRH into the portal circulation. The purpose of these experiments was to study the interactions of these two neurotransmitters and their effect on the release of LHRH. Both in-vitro and in-vivo studies were conducted to ascertain the effects of the GABAergic and serotonergic systems on hypothalamic LHRH neurons. The pharmacological manipulation of GABA receptors was used to affect serotonergic release and metabolism, as well as LHRH release. The temporal relationship between neurotransmitter stimulation and changes in LHRH release was studied. The extent that the rat's hormonal state influences these systems was explored. The behavior was monitored in in-vivo experiments to determine the effect of the rat's state of arousal on these interactions. The relationship between GABA and serotonin in the hypothalamus, the extent to which E_2 affects this relationship, and the ability of GABA stimulation to inhibit the release of LHRH from the hypothalamus under different hormonal conditions was determined by these studies.

Figure 1:



Anatomical relationship of pertinent neurons within the hypothalamus. MPOA represents the medial preoptic area and ME represents the median eminence.

SPECIFIC AIMS

This research established the effect of GABA receptor stimulation on LHRH release from the hypothalamus, as well as the release of serotonin and its major metabolite 5-HIAA, and how the cyclic E_2 fluctuations seen during the estrus cycle affected this stimulation. Serotonergic input to the hypothalamus originates in the raphe nuclei. In the hypothalamus serotonergic systems interact with both GABA and LHRH neurons.(61) Local GABA neurons, as well as serotonergic axons, make synaptic contact with LHRH neurons in the medial preoptic area of the hypothalamus.(28) These anatomical relationships are illustrated in figure 1.

The major aim of this research was to determine the effect exerted by stimulation of the local hypothalamic GABA system on serotonergic axons originating in the raphe, and how these two neurotransmitter systems interact to influence LHRH release. Another aim of this research was to determine how the fluctuating E_2 levels throughout the estrus cycle affect these systems. In-vitro superfusion was used to determine the changes in release of LHRH, 5-HIAA, and 5-HT seen under different hormonal conditions, the effect of GABA on LHRH, 5-HIAA, and 5-HT release during specific hormonal conditions, the level of GABA stimulation needed to cause an effect on LHRH release, and the extent that the effect of GABA stimulation was mediated by specific receptor subtypes. The changes in serotonergic systems and LHRH release within the hypothalamus caused by GABA stimulation were also investigated with in-vitro superfusion techniques. In-vivo push-pull perfusion was used to confirm the results of the in-vitro studies. Specifically, the present study will:

1) Establish baseline release of LHRH, and serotonergic activity, under four different hormonal conditions to determine how E_2 and progesterone might affect both LHRH and serotonin systems.

a) Proestrus and estrus hormonal states in cycling rats were used to represent extremes in the E_2 and progesterone levels during the estrus cycle.

b) A model system of ovariectomized (OVX) and, ovariectomized and estrogen replaced ($OVXE_2$), rats was used to compare and contrast the effect of a lack of E_2, and a low constant E_2 level, in the plasma with the regular cycling rat.

c) The activity of the serotonergic system within the hypothalamus was evaluated during each of these different endocrine conditions.

2) Determine the effect of GABA on LHRH secretion from, and serotonin activity within, the hypothalamus.

a) The effect of GABA stimulation on LHRH release, and serotonin activity, during proestrus was used to show how rising E_2 plasma levels affect GABA stimulation.

b) The effect of GABA stimulation on LHRH release, and serotonin activity, in $OVXE_2$ rats was used to show how a low chronic E_2 plasma level can affect GABA stimulation.

c) Determine the level of GABA stimulation needed to cause an effect on LHRH release from the hypothalamus of $OVXE_2$ rats.

3) Determine which of the GABA receptor subtypes is responsible for the effects of GABA stimulation.

a) Muscimol and baclofen were used as specific $GABA_A$ and $GABA_B$ agonists, while bicuculine and the histidine ligand were used as specific $GABA_A$ and $GABA_B$ antagonists.

b) The experiments were conducted on $OVXE_2$ rats to determine which subtype of GABA receptor mediates the effect of GABA on LHRH release, and serotonin activity, seen in rats with this endocrine status.

4) Confirmation of the in-vitro effects of GABA drugs on LHRH release and 5-HT activity using in-vivo techniques in $OVXE_2$ rats.

a) GABA and a $GABA_A$ receptor agonist, muscimol, were used in these experiments in order to determine the effect of GABA stimulation with an intact CNS.

b) The rat's behavior was evaluated to determine the effect of GABA on their state of arousal.

GENERAL
MATERIALS AND METHODS

Animals:

Virgin female Holtzman rats (200-250 grams) (Harlan Sprague Dawley Inc., Indianapolis, IN.) were kept in a well ventilated room with the temperature maintained at $24 \pm 1^{o}C$ (photoperiod 0500-1900). The animals were allowed food and water ad libitum. For the first week after arrival they were given time to acclimate to the animal facility, and were not cycled.

In the morning, the animal's cyclicity was determined by checking the vaginal histology of each rat. The vaginal smears were examined under a microscope and the cell types present in the smear were noted. Smears composed predominantly of leucocytes were considered diestrus; a predominance of cornified epithelial cells signified estrus, while the presence of round nucleated epithelial cells indicated proestrus. This examination was repeated daily until at least two consecutive four or five day cycles were observed.

Once cyclicity was determined some of the animals were ovariectomized and seperated into two treatment groups, OVX and OVX E_2 treated. The animal was placed under light ether anesthesia, and a skin incision was made five millimeters caudal to the apex of the curve of the last rib. The muscle wall was cut and the ovary exteriorized. A thread was tied tightly around the fallopian tube before cutting it, in order to prevent unnecessary bleeding. The fallopian tube was cut and the ovary discarded. The muscle wall was sutured together and the skin incision was closed with staples. The procedure was then repeated on the animal's other side.

In the $OVXE_2$ treatment group estradiol-containing silastic capsules were implanted at least two days before the experiment. Two to three weeks after the ovariectomy, the animals were again placed under light ether anesthesia and a skin incision was made on the dorsal surface of the animal's neck. A silastic capsule

containing 150 micrograms of estradiol per milliliter (ml) of sesame oil was implanted subcutaneously (82). The skin incision was then closed with surgical staples.

High performance liquid chromatography (HPLC):

Two HPLC sytems (Bioanalytical Systems, Inc. West Lafayette, IN) were used, the one for the push-pull perfusion and the dose response superfusion experiments (LC-300 series HPLC) and the other for superfusion and microdialysis experiments (BAS-400 series HPLC). The BAS-400 is a late model with many improvements in sensitivity and accuracy which was used in place of the LC-300. Once it became available we began using it since it was more sensitive. The LC-300 HPLC consisted of a PM-30A dual piston pump, a ten cm three micron phase II ODS column, a LC-4B amperometric detector with a glassy carbon electrode, and a Rheodyne 7125 injector. The BAS-400 HPLC consisted of a PM-48 pump, a LC-22A temperature controller, a one and a half cm seven micron ODS precolumn, a ten cm three micron phase II ODS column, two LC-4B amperometric detectors with glassy carbon electrodes, and a Rheodyne 7125 injector.

The LC-300 HPLC was run at a flow rate of one ml per minute. The detector was set with an applied voltage of 0.65 volts with a sensitivity of two nanoamps (nA), and an offset current from two to six nA was used to bring the baseline into the proper range. The chart speed was set at one half cm per minute. The BAS-400 HPLC was run at a flow rate of one ml per minute. The detectors were set at applied voltages of 0.65 and 0.8 volts (V) with sensitivities of one and two nA respectively. An offset current of zero to two nA was needed for the 0.65 V applied voltage; however, an offset current of two to four nA was needed for the 0.8 V applied voltage, to bring the baselines into the proper range. The column temperature was kept at a constant 40^{o}C by the LC-22A temperature controller. The chart speed was set at 0.1 mm per second.

Two mobile phases were used during these studies, an acetate citrate buffer (pH 4.5), and a monochloroacetic acid buffer (pH 3.1). The LC-300 HPLC system used the acetate citrate buffer, while the BAS-400 HPLC system used the monochloroacetic acid buffer. The push-pull perfusion and the dose response superfusion experiments used the acetate citrate buffer. While the other superfusion and microdialysis experiments used the monochloroacetic acid buffer. The composition of the two mobile phases was:

Acetate Citrate- 11.5 gm Citric acid, 8.2 gm anhydrous Sodium Acetate, 1.2 gm NaOH, 2.1 ml glacial Acetic acid, 85 mg Sodium Octyl Sulfate, 140 ml Methanol, 1860 ml deionized water. Adjust pH to 4.5 with glacial acetic acid.

Monochloracetic acid- 24.56 gm monochloroacetic acid, 400 mg Sodium Octyl Sulfate, 400 mg NaEDTA, 90 ml acetonitrile, 1910 ml deionized water. Adjust pH to 3.1 with five normal NaOH.

After the mobile phase had been completely mixed, and all solids were completely dissolved, it was filtered. A 0.2 micron filter was used under vacuum to remove any particulates that may damage the HPLC apparatus. The solution was maintained under vacuum for several hours until all gas bubbles were removed from the solution. Once the solution was completely degassed, it was run through the HPLC. It was allowed to circulate at a low flow rate overnight, to equilibrate the system, before any samples were run.

The morning of the experiment, standards were injected to calibrate the peak heights. Standards were prepared daily from stocks kept frozen in plastic vials. Standard stock solutions consisted of two mg of the standard mixed with twenty ml of 0.1 normal $HClO_4$. One ml of this solution was diluted with 50 ml of $HClO_4$. Aliquots of this solution were then frozen in plastic vials. Ten microliters of each

standard that was being measured that day was pipetted into a plastic vial on the morning of an experiment. This tube was labelled 1000 picograms (pg) and deionized water (or buffer) was added to bring the final volume to 400 microliters. This vial was well mixed. Then, half of its volume was put into a vial labelled 500 pg and this vial had its volume brought up to 400 microliters. This serial dilution was continued until a 62.5 pg vial was created. The 62.5 pg vial was the first standard to be injected. The injections continued through the 250 pg standard. At this point the maximum deflection of the pen on the chart recorder had usually been reached.

Radioimmunoassay (RIA):

All LHRH measurements were made with an RIA that used an antibody developed by Dr. William Ellinwood of the Department of Physiology, School of Medicine, The Oregon Health Sciences University. The ^{125}I-LHRH was purchased from Hazelton Biotechnologies, Vienna, VA. Two reagents used in the assay were made previously and frozen until they were needed. These reagents are PBSG and NRS.

The PBSG is a phosphate buffered saline solution with one percent gelatin. The phosphate buffered saline solution was made by dissolving 143 gm of NaCl in 500 ml of deionized water, adding 1.75 gm of merthiolate (ethylmercurithiosalicylic acid sodium salt), adding one liter of half molar monobasic phosphate solution, and finally adding 2.6 liters of half molar dibasic phosphate solution. This mixture was diluted to 17.5 liters with deionized water. The pH was brought to 7.4 with five normal NaOH. Next, the one percent gelatin was added. One gram of gelatin was weighed and it was dissolved in one liter of the solution while heating it.

The NRS solution used the same phosphate buffered saline solution as a stock solution. 18.612 gm of NaEDTA (ethylenedintrilotetracetic acid disodium salt), three ml of normal rabbit serum (NRS), and 800 ml of the solution were mixed. The pH

was brought up to 7.4 with five normal NaOH. The volume was brought up to one liter with the phosphate buffered saline solution. The NRS solution was used to dilute the antibody.

The assay was run over a five day period, and all procedures were carried out on ice. The five day length of the assay was empirically chosen to maximize the assay's sensitivity. On the first day the standards and samples were added to the tubes. All of the tubes, except for the total count and nonspecific binding tubes, had antibody added to them. On the second and fourth days, while sitting in the refrigerator, the tubes were shaken once. On the third day the ^{125}I-LHRH was added to the tubes. On the fifth day all tubes (except the total count tubes) had ice cold alcohol added to precipitate the Ag-Ab complexes. The tubes were then spun on the centrifuge, and the alcohol was decanted into the radioactive waste. Tubes were allowed to air dry in the fume hood before they were counted.

The standard curve used LHRH obtained from Sigma Chemical Company, St. Louis, MO. The concentrations of LHRH in the tubes range from 0.2 pg to 100 pg. Each tube in the assay (except the total count and nonspecific binding tubes) received fifty microliters of antibody dissolved in NRS, which gave a final dilution of the antibody of 1:400,000. The nonspecific binding tubes received fifty microliters of NRS solution without any antibody. The ^{125}I-LHRH was dissolved in PBSG and every tube received fifty microliters. The total count tubes had between 5000-6000 counts per minute when the tubes were counted. The sample tubes had a maximum of 200 microliters of sample added, and a minimum of 100 microliters of PBSG added.

Superfusion experiments:

Four sets of in-vitro superfusion experiments were conducted. The superfusion method allows the examination of physiological interactions within isolated

hypothalamic tissue. The tissue remained viable for a period of from four to five hours while neurotransmitter and hormone release, as well as the effect of drugs on the tissue, was examined. The types of superfusion experiments conducted were control, GABA stimulation, dose response, and receptor subtype determination. These experiments determined the validity of the technique, as well as the effect of GABA on the tissue.

The superfusion apparatus was made from a medium sized plastic tub (32cm x 25cm x 14cm) according to the method of Levine and Ramirez (46). Four three ml syringes were cut in half and connected to four stainless steel eighteeen gauge needles, which were anchored in the floor of the tub using rubber connectors. Tubing ran from the needles to a Minipulse 2 pump, Gilson Electronics, Middleton, WI. Rubber gaskets constructed from the syringe plungers were used to define the chamber that contained the tissue. The tissue was carefully lifted up with smooth forceps using capillary action. The tissue was placed on a rubber gasket covered with glass wool inside the syringe and covered with another similar gasket, at all times being careful not to squeeze the tissue and further tramautize it.

Dried, and then humidified, air was infused into the top of the syringe to aerate the tissue. The tissue was constantly bathed in Krebs-Ringer-Phosphate (KRP) buffer. The air was also used to drive the sample out of another tube connected to the top of the syringe. The sample flowed through this tube into a plastic test tube on ice. The KRP buffer contained 120.3 millimolar (mM) NaCl, 4.8 mM KCl, 2.58 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, and 12 mM phosphate (pH 7.4). The buffer also contained one percent bovine serum albumin. A high potassium (60 mM) KRP buffer was used in the last half hour of the superfusion. This buffer had 64.8 mM NaCl and 60 mM KCl as the only concentration differences from the other KRP buffer. The flow rate was set at 50 microliters/min.

Once the sample was collected it was acidified and then neutralized to deactivate any peptidases that might break down the LHRH. First, fifty microliters of one normal

HCl was added, then thirty microliters of 0.1 M phosphate buffer was added, and finally twentyfive microliters of two normal NaOH was added. The pH of all tubes was checked to make sure it was between 6.5-7.5 before they were frozen.

The tissue was obtained in the morning around 1000 hours. The rats were killed with a guillotine and the brain was quickly removed. The tissue used in all experiments contained the medial basal hypothalamus and the preoptic area, including the suprachiasmatic nucleus (MBH-POA-SCN). The brain was laid on a paper towel with the ventral side up. Cuts were made, one millimeter rostral to the optic chiasm, laterally along the hypothalamic sulci, immediately rostral to the mamillary bodies, and dorsally along the floor of the third ventricle, using a pair of fine iris scissors. The tissue was immediately placed in ice cold KRP buffer.

The entire operation was completed within ten minutes. Four rats were used in each experiment and the tissue collection procedure was accomplished within an hour. The tissue was handled as gently as possible throughout the procedure to maximize its viability. Samples were collected every ten minutes in all superfusion experiments, and all samples were from 400-800 microliters with most samples being about 500 microliters. The first hour of collection, of all superfusion experiments, was discarded to allow the system to reach equilibrium.

Push-pull perfusion experiments:

The push-pull perfusion (PPP) technique was used to examine the effect of local GABA stimulation on the hypothalamic release of neurotransmitters and hormone. All of these studies were done on $OVXE_2$ rats, and the median eminence (ME) was the area perfused in all rats (a few rats had their suprachiasmatic nucleus (SCN) cannulated in order to practice the procedure, but these rats were not included in the results). The push-pull perfusion technique allowed the measurement of synaptic release within the

median eminence area of awake freely behaving rats. The results of these studies were used to corroborate the in-vitro superfusion experiments.

Each cannula used in the push-pull perfusion (PPP) experiments was made by the author. The outer cannula was made from a 24 gauge stainless steel needle that was cut to the proper length for the area being investigated (9 mm for suprachiasmatic nucleus and 13 mm for median emminence). When implanted the outer cannula contained a stylette which extended 0.5 mm beyond the end of the cannula. The stylette was made from 43 mm of 33 gauge stainless steel tubing. Both ends of the tubing were occluded. A one ml plastic syringe was cut at the 0.05 ml line, and small indentations were made on the inside of the syringe with a sharp probe. A small portion (4-5 mm) of rubber tubing (0.05 cc/m) was used to position the tubing in the syringe. The 33 gauge tubing was pushed down until 0.5 mm was sticking beyond the end of the stainless steel syringe. Then the plastic syringe was filled with quick drying epoxy resin, being careful not to overflow the sides. Once the epoxy hardened the stylette was removed and any rough edges were smoothed over so that the animals couldn't grab onto it when the cannula was implanted.

The inner cannula assembly used a one ml plastic syringe, prepared similarly to the stylette, for it's base. Once again 33 gauge stainless steel tubing was used, although this time, care was taken when cutting it to ensure that both ends remained open. This tubing was for the push portion of the assembly, and was five to six cm long. Another shorter (four to five cm) length of 29 gauge stainless steel tubing was used for the pull portion of the assembly. Once again care was taken during cutting to insure that both ends of the tubing remained open. Also a small length of rubber tubing was again used to position the 29 and 33 gauge tubing in the syringe. The 29 gauge tubing was positioned so that it protruded slightly (0.1 mm) from the bottom of the plastic syringe. The inner assembly was then placed in the outer cannula. The 33

gauge tubing was positioned so that 0.33 mm protruded from the bottom of the outer cannula.

The plastic syringe was partially (one half to three quarters) filled with epoxy. This was allowed to harden while appropriate lengths (80-100 cm) of PE 20 tubing were cut. Once the epoxy had hardened, the inner assembly was removed from the outer cannula. The PE 20 tubing was then glued onto the 33 and 29 gauge tubings using epoxy. This was allowed to harden, and if necessary another coat of epoxy was applied to ensure that the assembly was sturdy enough. The rat would attempt to remove the assembly at first, but if it was properly constructed the rat would not be able to damage it.

Seven to ten days prior to the experiment the outer cannula and stylette were implanted. The surgery was done under ketamine-acepromazine (10:1) anesthesia. The ketamine solution was at a concentration of 100 mg/ml, and the acepromazine concentration was 10 mg/ml. The dosage was 100 microliters/100 gm body weight. Atropine (10 mg/ml) was given at the same dosage to inhibit mucous secretion. The rat was also administered 0.2 ml of penicillin G (50,000 units/ml) to prevent infections. A stereotaxic apparatus was used for the surgery. The ear bars were positioned, being very careful to ensure they held the head solidly in place. The mouth of the rat was placed so that the incisors were over the mouthpiece, and the nose bar was secured tightly over the bridge of the nose. Once the rat was correctly positioned in the stereotaxic apparatus, surgery could begin.

An incision was made from a point between the eyes to the base of the skull. The fascia was scraped away from the skull and small hemostats were used to hold it back. An epinephrine (one mg/ml) solution was applied to cause capillary constriction and minimize bleeding. Bone wax was rubbed into the skull to prevent bleeding through the bone. The bregma was located and used to provide a zero point for the cannula. The cannula was then moved to the proper coordinates for the implantation

site and this spot was marked on the skull. For the suprachiasmatic nucleus (SCN), those coordinates were anterior +0.15 cm and lateral +0.025 cm; while for the median eminence (ME), the coordinates were anterior -0.05 cm and lateral -0.05 cm. Now the cannula was moved away and three holes were drilled into (but not through) the skull. The holes were placed so that there was ample room for the cannula to be implanted. Three stainless steel machine screws were then screwed into these holes.

A hole was drilled through the skull, at the point marked earlier, for the implantation of the cannula. The cannula was then lowered until it touched the dura mater covering the brain. The vertical coordinate was noted and the proper amount (0.838 cm for SCN and 0.98 cm for ME) was subtracted for the area desired. The cannula was then lowered to that depth, and dental acrylic was used to cement the cannula to the skull. The dental acrylic was allowed to harden, and the cannula was then loosened from the holder. Another coating of dental acrylic was applied, taking care to ensure that no ragged edges were left for the rat to grab onto. The rat was kept warm until it awakened, at which time it was taken back to the animal facility.

On the morning of the experiment the rat was removed from the animal facility and the corresponding inner cannula assembly was implanted. The inner cannula assembly was first balanced on the push and pull pumps. Two Minipulse 2 pumps (Gilson Electronics, Middleton WI) were used as push and pull pumps. The assembly was connected to a swivel which was connected to the pumps. The pumps were balanced by maintaining a bubble on the tip of the assembly holder for at least ten minutes. The flow rate was 20 micro liters/min. The rat was removed from the cage and placed under a light ether anesthesia. The stylette was removed and the inner cannula assembly was put in its place. The tubing was closely watched for the presence of bubbles. If bubbles were seen, all connections were checked for clogs.

The control perfusion used an artificial cerebrospinal fluid (aCSF) to maintian physiological conditions at the site of perfusion. The composition of the aCSF was:

aCSF- 120.3 mM NaCl, 4.8 mM KCl, 2.58 mM $CaCl_2$, 1.2 mM $MgSO_4$, 12 mM phosphate

These salts were dissolved in deionized water and the pH was adjusted to 7.4 with either 0.1 normal HCl or NaOH. The drugs were dissolved in aCSF that had 0.1% ethylene glycol added to aid in dissolving the drugs. All of the drugs were perfused in a final concentration of 10^{-6}M.

The brains of the rats used in these experiments were removed and examined histologically. The rat was again anesthetized with the 10:1 ketamine-acepromazine, except that this time the dose was 250 microliters/100 gm body weight. An incision was made into the abdomen and the rib cage was opened. A cardiac puncture was performed and the rat's blood was washed out with 0.9% saline. A 10% formalin solution was then infused for several minutes. Once the body had ceased twitching, the infusion was stopped and the head removed. The brain was carefully removed from the skull and placed in a scintilation vial containing 10% formalin. The vials were kept in the refrigerator for several weeks before the brains were sectioned.

Sectioning was done on a freezing microtome at -26°C. The brains were placed on a paper towel with the ventral side facing upward. The rostral cut was made just anterior to the optic chiasm, while the caudal cut was made just posterior to the ME. This section was then mounted (rostral side up) on a chuck from the microtome. Forty micron sections were cut from the tissue and thaw mounted on subbed slides (six sections per slide). The slides were then air dried for 24 hours before being stained with cresyl violet. After the slides were stained, they were treated with histoclear, and a permanent coverslip was applied with permount. The slides were allowed to dry for a day, before they were examined to determine the location of the cannula tip.

SPECIFIC EXPERIMENTAL PROCEDURES

Superfusion experiments:

1) The control experiments were done to examine how different hormonal conditions affected the tissue, as well as to validate the viability of the tissue. The paradigm used in these experiments consisted of a four hour incubation with KRP buffer, followed by a half hour of high K^+ KRP buffer. This allowed the release of LHRH, 5-HIAA, and 5-HT from hypothalamic tissue during this time period to be quantitated with RIA and HPLC. The amount of these chemicals that were released during the four endocrine states (proestrus, estrus, $OVXE_2$, and OVX) was compared in these experiments.

I----------KRP----------I-60 mM K^+-I

FOUR HOUR CONTROL PERIOD — POTASSIUM STIMULATION

2) The GABA stimulation experiments were designed to examine the effect of 0.1 mM GABA on the tissue. Two hormonal states (proestrus and $OVXE_2$) were chosen for these experiments to contrast the effect of GABA stimulation with high, rising plasma E_2 levels and low, chronic plasma E_2 levels. The paradigm for these experiments consisted of two hours of KRP buffer superfusion, followed by two hours of KRP buffer containing 0.1 mM GABA (Sigma Chemical Co., St. Louis, MO), and a final half hour of superfusion with high K^+ KRP. In these experiments the release of LHRH, 5-HIAA, and 5-HT was quantitated by RIA and HPLC. The amount of these chemicals released during periods of GABA stimulation, and during periods without GABA stimulation, was contrasted in these experiments.

I----------KRP----------I----------GABA----------I-60 mM K^+-I

TWO HOUR CONTROL PERIOD — TWO HOUR GABA STIMULATION — POTASSIUM STIMULATION

3) The dose response experiments determined the degree of GABA stimulation needed to cause an effect. Dose response experiments were done with GABA, and the $GABA_A$ receptor agonist muscimol. The concentrations of GABA that were used are 10^{-6} M, 10^{-5} M, 10^{-4} M, while the concentrations of muscimol were 10^{-7} M, 10^{-6} M, 10^{-5} M. The protocol followed by all of the dose response experiments was: four samples were collected with just KRP buffer, six samples were collected with each concentration of the drug (starting from lowest and going to highest), another six samples were collected with just KRP buffer, and finally three samples were collected with high K^+ KRP buffer. The data collection lasted for five hours and ten minutes. These samples were all analyzed by RIA for LHRH data.

I----KRP----I-----10^{-6}M----I-------10^{-5}M------I------10^{-4}M--------I----------KRP-----I---60mM K^+----I

CONTROL PERIOD	10^{-6} GABA DOSE		10^{-5} GABA DOSE		10^{-4} GABA DOSE	CONTROL PERIOD	POTASSIUM STIMULATION

I----KRP----I-------10^{-7}M----I-------10^{-6}M------I-------10^{-5}M-------I--------KRP------I---60mM K^+----I

CONTROL PERIOD	10^{-7} MUS. DOSE		10^{-6} MUS. DOSE		10^{-5} MUS. DOSE	CONTROL PERIOD	POTASSIUM STIMULATION

4) The receptor subtype experiments attempted to discover which subtype of GABA receptor was responsible for which portion of the effect of GABA stimulation. The $OVXE_2$ hormonal state was the only one used in these experiments to allow the comparison of the different receptor subtype stimulation under constant plasma E_2 levels. All of the drugs used in these experiments were made up in 0.1 mM concentrations. The GABA-A receptor agonist muscimol, and the GABA-A receptor antagonist bicuculine were both purchased from Sigma Chemical Company, St. Louis, MO. The GABA-B receptor agonist baclofen was a gift from CIBA-GEIGY Corp., Summit, NJ. The histidine ligand that was used as a GABA-B antagonist was synthesized by Dr. Roy Williams in the Old Dominion University Department of Chemical Sciences. The protocol used was the same one used in the GABA stimulation

experiments. The only difference was that the particular drug specific for the receptor being investigated replaced GABA. All of the experiments were carried out with agonist in two tubes and antagonist in two seperate tubes. The tubes containing the agonist and antagonist were switched between experiments. The samples were also analyzed with both HPLC and RIA for 5-HIAA and LHRH data.

I----------KRP----------I----------DRUG----------I------60 mM K^+------I

TWO HOUR CONTROL PERIOD	TWO HOUR DRUG STIMULATION	POTASSIUM STIMULATION

Push-pull perfusion experiments:

A total of seventeen rats were used in the ME PPP experiments.

1) Five rats were used as control (two were four hour perfusions and three were eight hour perfusions).

I----------aCSF----------I

PERFUSION WITH ARTIFICIAL CEREBROSPINAL FLUID

2) One rat was perfused for four hours with vehicle (0.1% ethylene glycol) preceded by four hours of control.

I----------aCSF----------I----------VEHICLE----------I

CONTROL PERFUSION 0.1% ETHYLENE GLYCOL PERFUSION

3) Five rats were perfused with GABA. Four hours of GABA preceded by four hours of control.

I----------aCSF----------I----------GABA----------I

CONTROL PERFUSION 10^{-6}M GABA PERFUSION

4) Six rats were perfused with muscimol. Five for four hours and one for three hours.

I----------aCSF----------I----------MUSCIMOL----------I

CONTROL PERFUSION 10^{-6}M MUSCIMOL PERFUSION

In the experiments that had drugs perfused the drugs were perfused during the last half of the experiment. This procedure allowed the evaluation of the drug's effects within each individual animal.

Behavioral studies:

The rats in the ME PPP experiments had their behavior evaluated. The behavioral evaluation was used to determine the rat's state of arousal during the experiment, and how this level of arousal may be changed with drug treatment. A response-reactivity scale was constructed and used to evaluate their behavior. In all experiments it was administered once an hour. Three stimuli were used, a puff of air on the rat's back, a touch on the nose with a cotton swab, and finally an attempt was made to pick up the rat. The rat's score depended on the severity of their reaction to the stimulus. A score of zero indicated that the rat made no response to that stimulus. A three point scale was used in evaluating all responses to the stimuli. For the puff of air a one indicated an orienting response, a two indicated that the rat was startled, and a three was given if the animal jumped. The touch with a cotton swab was scored a one if the rat orients to the swab, a two was given when the rat sniffed the swab, and a three indicated that the rat tried to taste or bite the swab. The attempt to pick up the rat was given a one if the rat vocalized, a score of two showed that the rat struggled, a three signified that the rat ran when the hand approached. These three scores were then added to give an index of the rat's state of arousal.

Statistics:

Parametric tests were used to determine significance of all data, except the behavioral scores. The behavioral scores were evaluated using the nonparametric Wilcoxon signed rank test. The parametric tests used were ANOVA and the paired T-test. The computer program PULSAR was used to evaluate the pulsatility in much of

the data. PULSAR is a program designed to identify peaks in a series of points. It was written to assist in the analysis of episodic hormone secretion. It was used in the control and GABA superfusion experiments, as well as the ME PPP experiments, to evaluate the neurotransmitter and LHRH data. The data from the dose response superfusion experiments was converted to percent of control values, and then significance was determined by a paired T-test. The control and GABA superfusion experiments, along with the GABA subtype superfusion experiments, were evaluated with both ANOVA and paired T-tests. The PPP experiments were evaluated by ANOVA, while the data from the microdialysis experiments was converted to percent of control before a paired T-test was done.

RESULTS

A) IN-VITRO SUPERFUSION EXPERIMENTS:

The superfusion experiments were done with virgin female rats. The rats were either, cycling animals in proestrus and estrus, or their ovaries had been removed. The ovariectomized group was further divided into a group that received estradiol ($OVXE_2$) and one that did not (OVX). Four types of superfusion experiments were run: control, GABA stimulation, dose response, and receptor subtype determination. These experiments allowed the determination of the effect of GABA on isolated hypothalamic tissue from female rats.

1) Control:

All of the control experiments were done with eight rats. Four endocrine states were used (proestrus, estrus, $OVXE_2$, and OVX), and LHRH as well as 5-HT and 5-HIAA data was obtained. These states allowed the examination of the release of these chemicals under a variety of hormonal conditions. The proestrus and estrus rats showed the changes in release during the estrous cycle, while the OVX and $OVXE_2$ rats showed the changes in release caused by absence of E_2 in the plasma or the presence of low constant plasma E_2 levels.

LHRH release:

The greatest amount of LHRH released during any of the superfusion experiments was seen during the proestrus experiments (fig. 2). Although the release peaked in the second hour and fell for the third and fourth hours, there were no significant differences between the first four hours of the experiment. However, each rat's last half hour of potassium stimulation was significantly different from at least part of the first four hours. The LHRH release during estrus was significantly lower than it was during proestrus (fig. 3). However, the same general pattern was seen, with no

Figure 2: LHRH release during the proestrus control experiment. The mean level of LHRH released during each ten minute period is shown for four hours of superfusion with KRP buffer. Eight rats were used in this experiment.

FIG. 2
MEAN LHRH RELEASE IN PROESTRUS
N=8

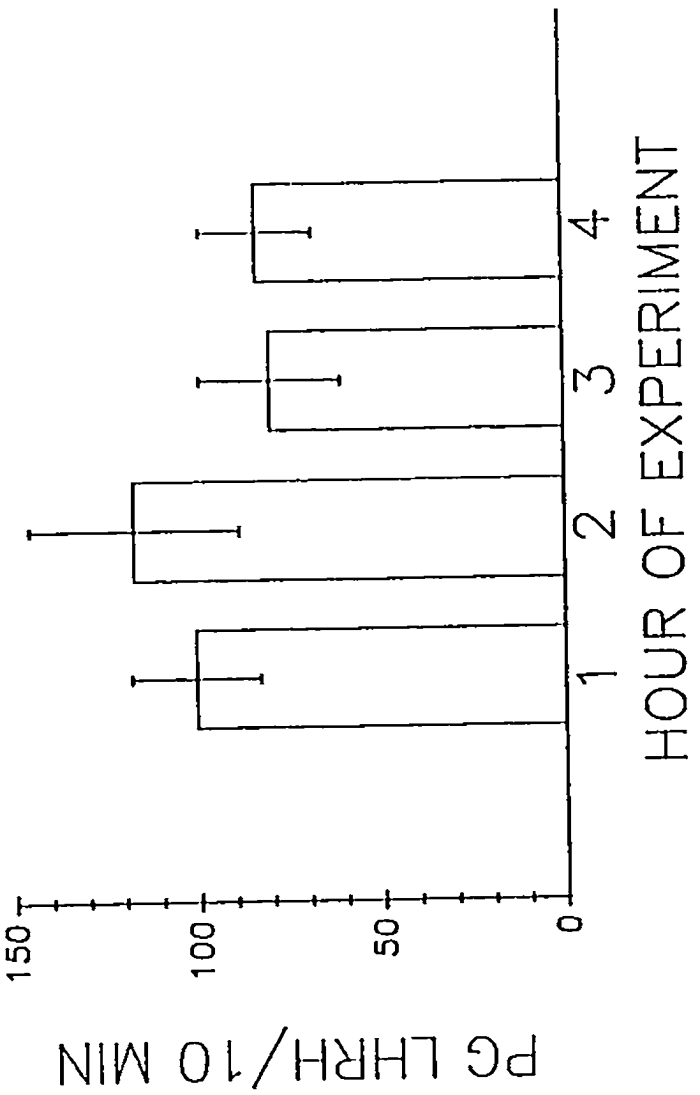
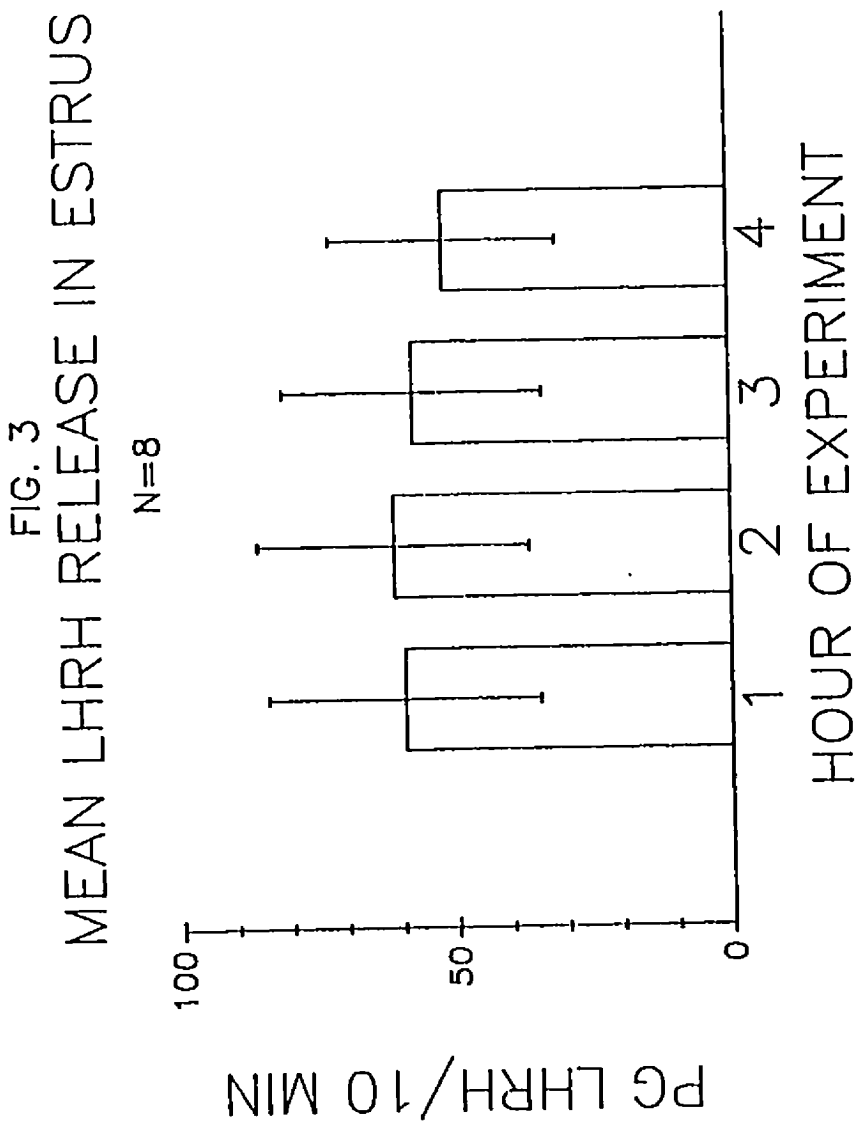


Figure 3: LHRH release during the estrus control experiment. The mean level of LHRH released during each ten minute period is shown for four hours of superfusion with KRP buffer. Eight rats were used in this experiment.



significant differences between the hours of control superfusion. Only two of the estrus rats had a significantly different amount of LHRH released during the potassium stimulation. The amount of LHRH released during the OVX and $OVXE_2$ experiments was much smaller than that released during the proestrus or estrus experiments (fig. 4). There were no significant differences between the hourly means. There was also no significant difference between the amount of LHRH released during the OVX or $OVXE_2$ experiments.

Table 1: Average LHRH release (n=8) (pg/10 min) during the control superfusion experiments. Mean ± Standard Error

MEAN	PROESTRUS	ESTRUS	OVX	$OVXE_2$
First hour	101±17	59±25	2.9±1.7	1.7±0.75
Second hour	117±28	61±25	1.6±1.0	2.0±1.1
Third hour	97±18	58±24	1.7±1.1	0.77±0.28
Fourth hour	79±15	52±21	1.4±0.82	0.67±0.24
Overall	99±8	58±2	1.9±0.34	1.3±0.33

Two way ANOVA for hormonal state and hour of experiment. $F_{3,28}=36.64$ for significant difference ($p<.05$) in the hormonal state. Duncan's test shows that proestrus is significantly different ($p<.05$) from estrus, and that OVX and $OVXE_2$ are similar to each other while being significantly different ($p<.05$) from proestrus and estrus.

Serotonin and 5-HIAA release:

Small amounts of serotonin were released during the proestrus experiments (fig. 5). During proestrus serotonin release was charecterized by periods that were below the level of detection for serotonin. Two rats were the only ones that had more than ten data points for the entire four and a half hour period. The 5-HIAA release during proestrus declined during the course of the experiment (fig.6). The release during the first hour of superfusion was significantly higher than during the third and fourth hours. The amount of serotonin released during the estrus experiments was significantly greater than the amount released during the proestrus experiments (fig. 7).

Figure 4: LHRH release during the OVX and $OVXE_2$ control experiments. The mean level of LHRH released during each ten minute period is shown for four hours of superfusion with KRP buffer. No significant differences were seen between the amounts of LHRH released during each hormonal state. Eight rats were used in both of these experiments.

FIG. 4

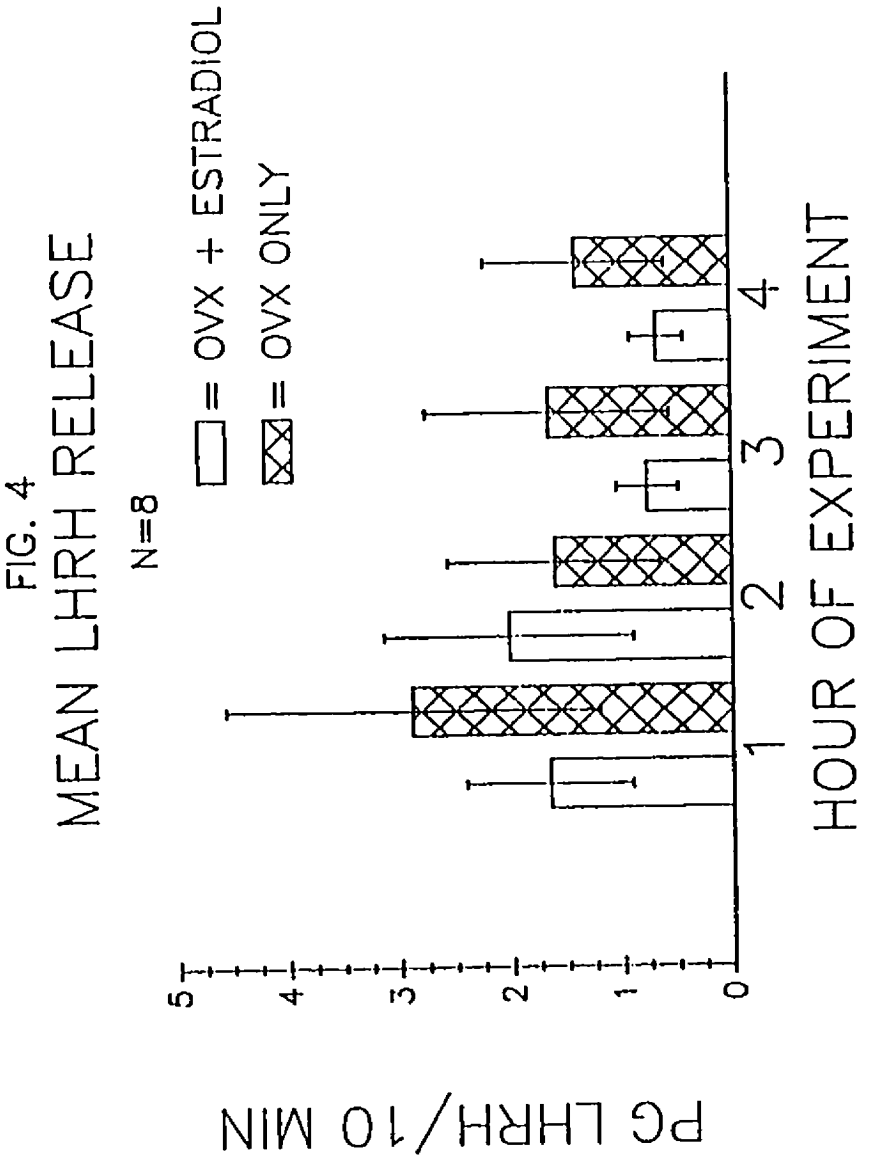


Figure 5: Serotonin release during the proestrus and OVX control experiments. The mean level of serotonin released during each ten minute period is shown for four hours of superfusion with KRP buffer. No significant differences were seen between the amounts of serotonin released during both hormonal states. Eight rats were used in both of these experiments.

FIG. 5

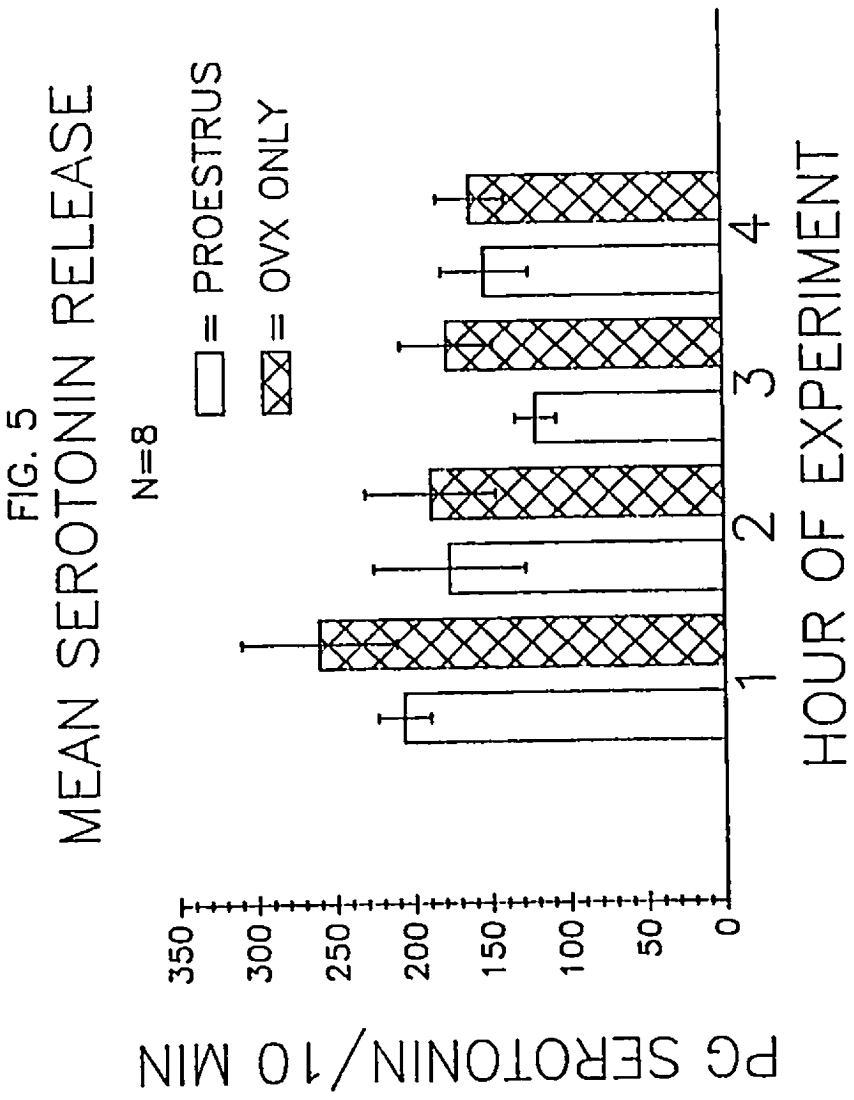


Figure 6: 5-HIAA release during the proestrus control experiment. The mean level of 5-HIAA released during each ten minute period is shown for four hours of superfusion with KRP buffer. The amount released during the third and fourth hours is significantly lower than the amount released during the first hour. Eight rats were used in this experiment.

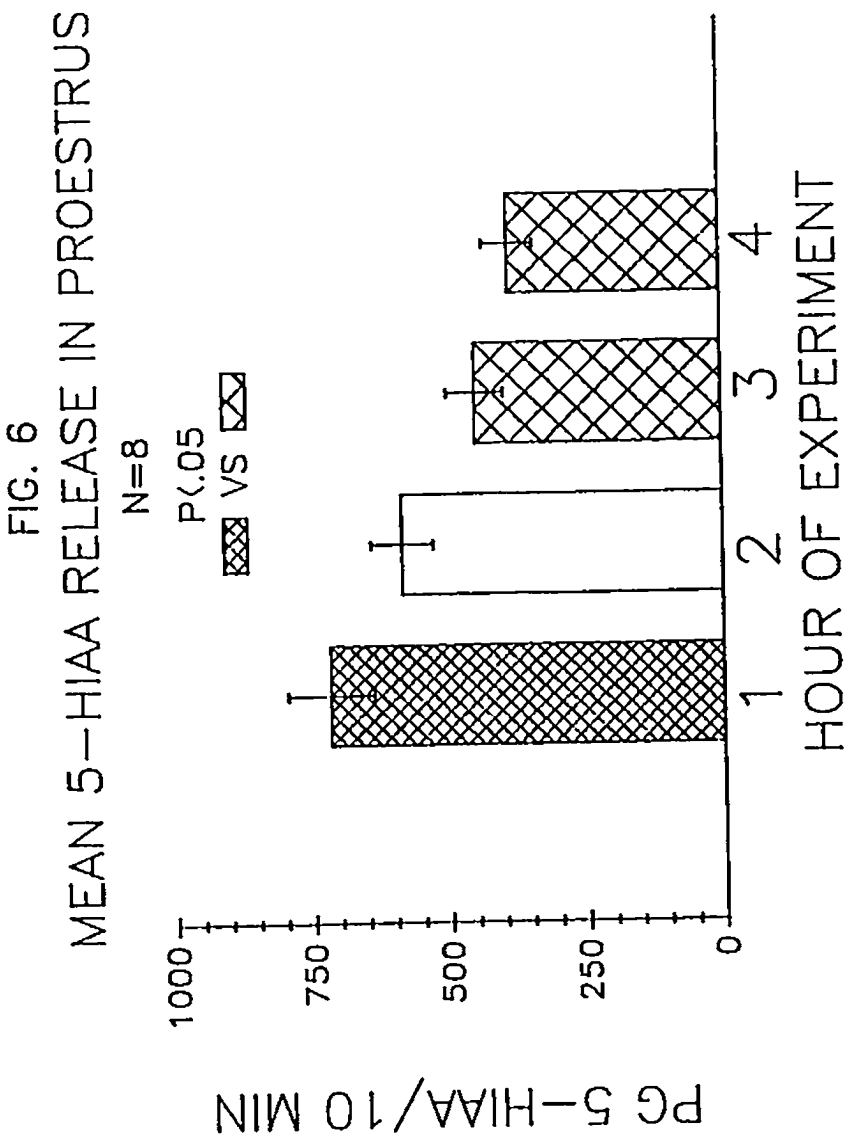
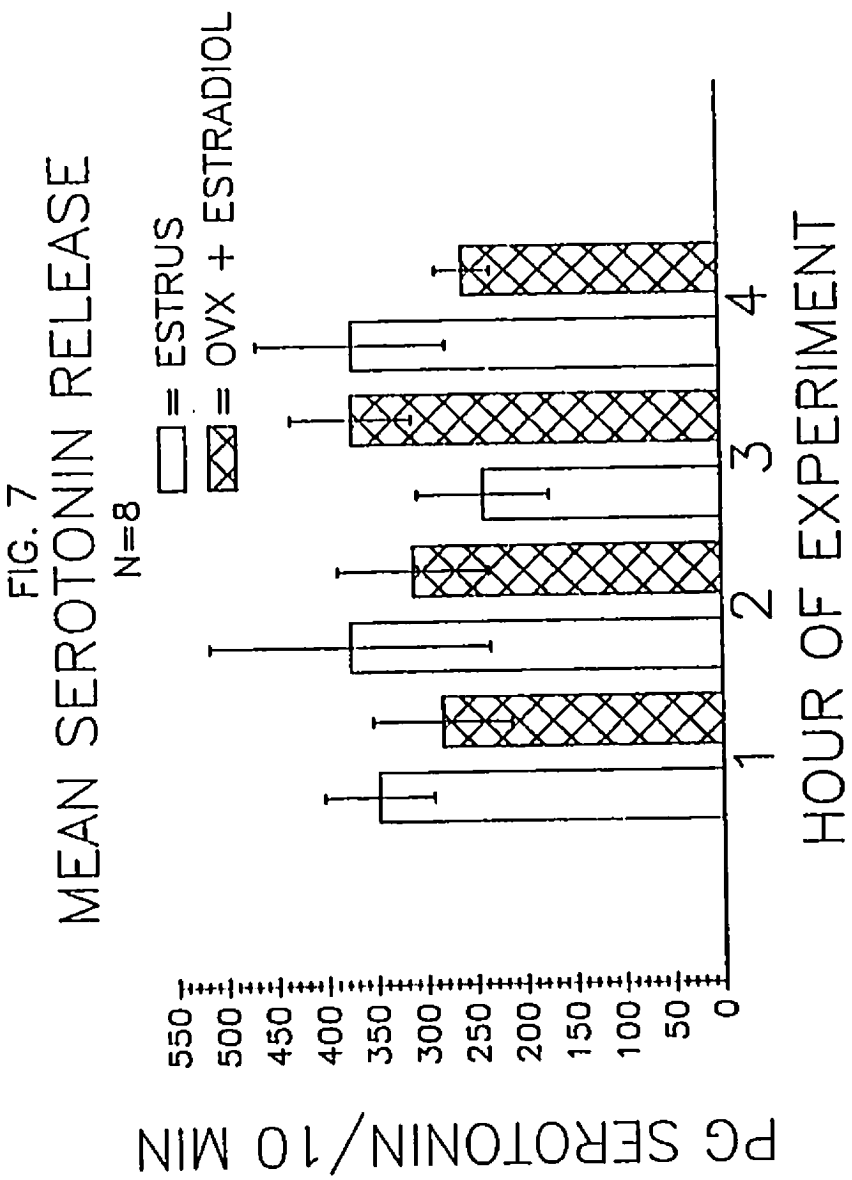


Figure 7: Serotonin release during the estrus and $OVXE_2$ control experiments. The mean level of serotonin released during each ten minute period is shown for four hours of superfusion with KRP buffer. No significant differences were seen between the amounts of serotonin released during both hormonal states. Eight rats were used in these experiments.



However, the standard error of the serotonin means was much greater during the estrus experiments than during the proestrus experiments.

The 5-HIAA release followed the same pattern in the $OVXE_2$ experiments that it did in the proestrus experiments. The release during the first hour was significantly higher than the release during the third and fourth hours (fig.8). The amount of serotonin released was higher during the $OVXE_2$ experiments than it was during the OVX experiments (figs. 5 and 7). The amount of serotonin released during the $OVXE_2$ experiments was similar to the amount of serotonin released during the estrus experiments, while the serotonin data from the OVX experiments was similar to the serotonin data from the proestrus experiments. The $OVXE_2$ experiments released significantly more serotonin than was released during the proestrus experiments.

Table 2: Average 5-HT release (n=8) (pg/10 min) during the control superfusion experiment. Mean ± Standard Error

MEAN	PROESTRUS	ESTRUS	OVX	$OVXE_2$
First hour	206±17	347±55	260±49	283±70
Second hour	176±49	375±141	188±42	311±76
Third hour	120±13	240±67	177±30	372±61
Fourth hour	152±28	371±94	161±22	258±27
Overall	164±18	333±32	197±22	306±25

Two way ANOVA for hormonal state and hour of the experiment. $F_{3,28}=8.39$ for significant difference ($p<.05$) in the hormonal state. Duncan's test showed that the estrus and $OVXE_2$ hormonal states were similar, and the proestrus and OVX hormonal states were similar. The estrus and proestrus hormonal states were significantly different ($p<.05$), while the $OVXE_2$ and OVX hormonal states were significantly different ($p<.05$).

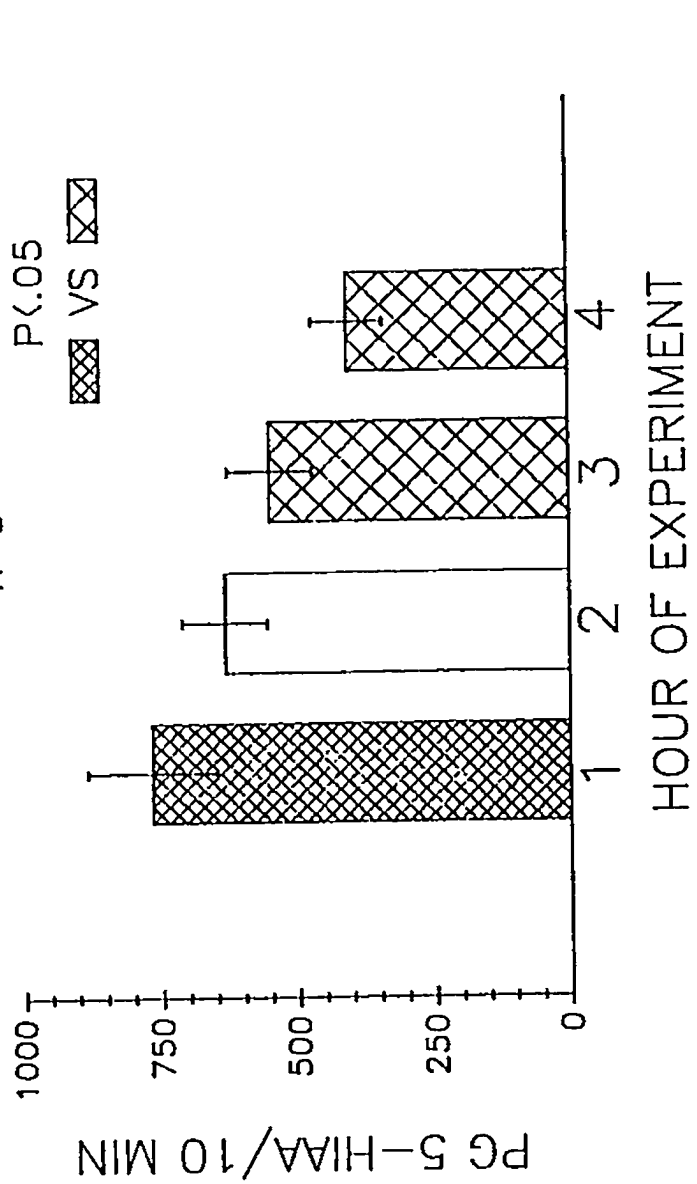
Table 3: Average 5-HIAA release (n=8) (pg/10 min) during the control superfusion experiment. Means ± Standard Error

MEAN	PROESTRUS	ESTRUS	OVX	$OVXE_2$
First hour	717±79	794±151	904±222	766±117
Second hour	585±56	687±136	808±275	632±78
Third hour	449±52	561±123	682±267	549±78
Fourth hour	386±46	516±85	564±245	405±65
Overall	534±74	640±63	740±74	588±76

Figure 8: 5-HIAA release during the $OVXE_2$ control experiment. The mean level of 5-HIAA released during each ten minute period is shown for four hours of superfusion with KRP buffer. The amount released during the third and fourth hours is significantly lower than the amount released during the first hour. Eight rats were used in this experiment.

FIG. 8

MEAN 5-HIAA RELEASE IN OVX + ESTRADIOL



Two way ANOVA for hour of experiment and hormonal state. $F_{3,28}=3.55$ for a significant difference ($p<.05$) in the hour of the experiment. Duncan's test showed that the first hour was significantly different ($p<.05$) from the third and fourth hours. Subsequent one way ANOVA showed that these hours were only significantly different ($p<.05$) in the proestrus and $OVXE_2$ hormonal states.

2) GABA stimulation:

Two states charecterized by high and low E_2 levels, proestrus and $OVXE_2$, were used for the GABA stimulation experiments. These two states allowed the examination of the effect of GABA during conditions of rising, high plasma E_2 levels and constant, low plasma E_2 levels. The LHRH data from eight proestrus rats showed no significant difference between GABA stimulation and control superfusion (fig. 9). However, The LHRH data from six $OVXE_2$ rats showed a significant decrease during GABA stimulation (fig. 10). No significant differences in the release of 5-HIAA or 5-HT were observed during GABA stimulation.

Table 4: Average LHRH release (n=8 for proestrus and n=6 for $OVXE_2$) (pg/10 min) during the GABA stimulation experiment. Boldface indicates 0.1 mM GABA was being superfused. Means $\pm$ Standard Error

MEAN	PROESTRUS	$OVXE_2$
First hour	2.5±1	7.0±5
Second hour	2.8±2	8.4±5
Third hour	**2.9±2**	**5.7±3**
Fourth hour	**3.1±1**	**5.0±3**
Overall	2.8±0.1	6.5±0.7

One way ANOVA for GABA stimulation done on $OVXE_2$ data $F_{5,12}=23.46$. LHRH release was significantly ($p<.05$) decreased during the GABA stimulation. Proestrus ANOVA was not significant.

Table 5: Average serotonin release (n=8) (pg/10 min) during the GABA stimulation experiment. Boldface indicates 0.1 mM GABA was being superfused. Means $\pm$ Standard Error

MEAN	PROESTRUS	$OVXE_2$
First hour	556±82	376±63
Second hour	551±93	384±74
Third hour	**610±83**	**361±57**
Fourth hour	**573±80**	**392±50**
Overall	573±13	378±7

Figure 9: LHRH release during the proestrus GABA stimulation experiment. The mean level of LHRH released during each ten minute period is shown for the entire experiment. No significant difference is seen during the GABA stimulation. Eight rats were used in this experiment.

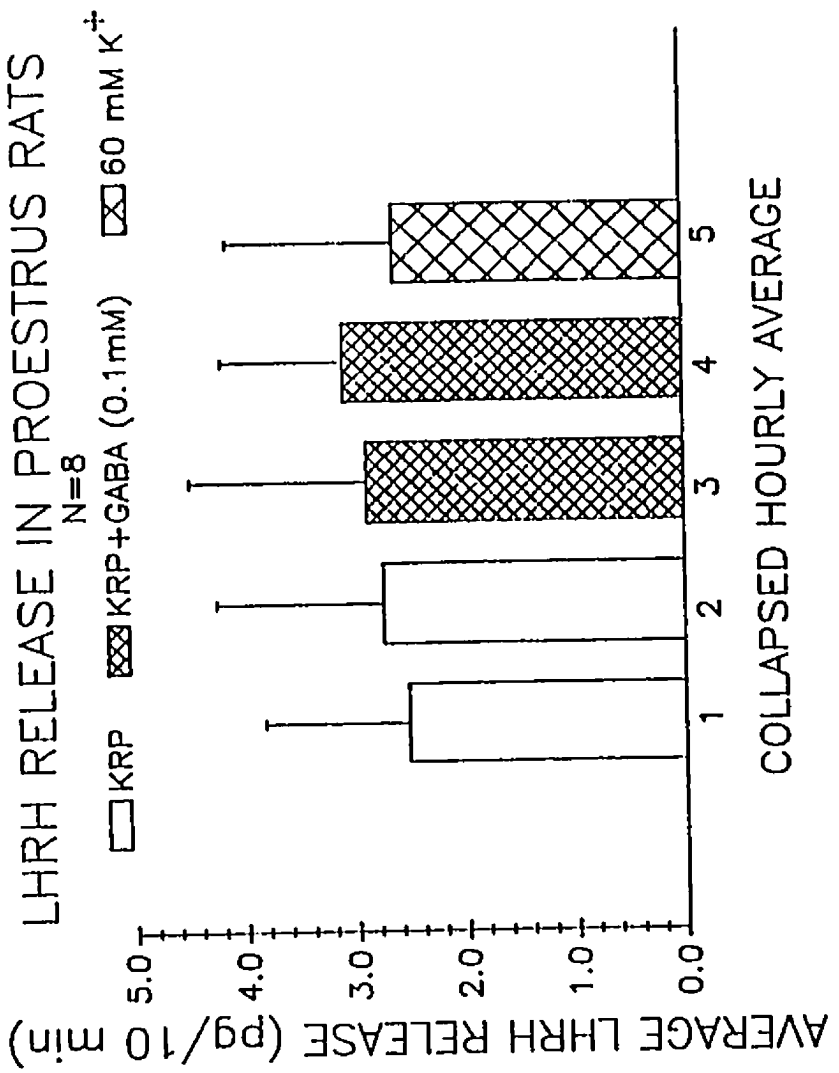
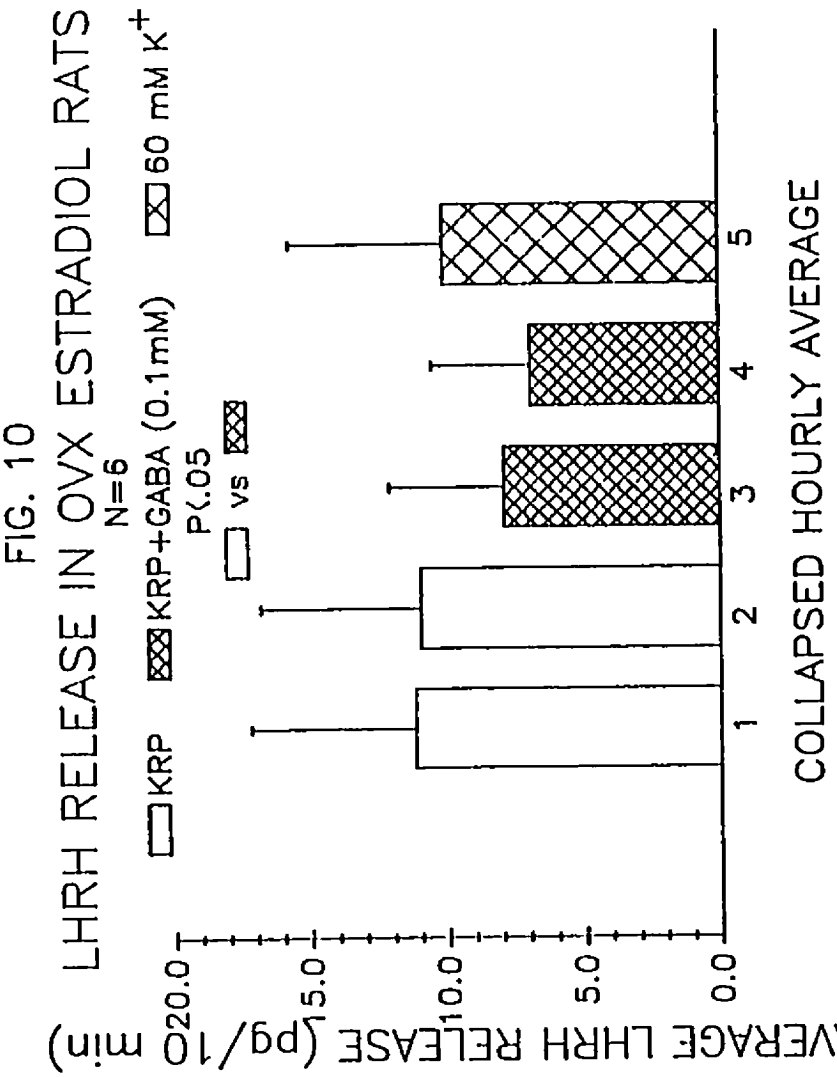


Figure 10: LHRH release during the $OVXE_2$ GABA stimulation experiment. The mean level of LHRH released during each ten minute period is shown for the entire experiment. The GABA stimulation significantly decreased the level of release. Six rats were used in this experiment.

FIG. 10



One way ANOVA for GABA stimulation was not significant.

Table 6: Average 5-HIAA release (n=8) (pg/10 min) during the GABA stimulation experiment. Boldface indicates 0.1 mM GABA was being superfused. Means ± Standard Error

MEAN	PROESTRUS	$OVXE_2$
First hour	959±45	864±133
Second hour	827±31	875±220
Third hour	736±58	817±162
Fourth hour	630±50	928±213
Overall	788±70	871±23

One way ANOVA for GABA stimulation was not significant.

3) Dose response:

Seven $OVXE_2$ rats were used in each of these experiments. The only data that was obtained in these experiments was the amount of LHRH released. The GABA experiments showed a significant difference between the 10^{-6}M concentration, and both the 10^{-4}M and 10^{-5}M concentrations (fig. 11). The muscimol experiments showed a significant difference between the 10^{-7}M and the 10^{-5}M concentrations (fig.12). Interestingly, the 10^{-7}M concentration shows slightly (but not significantly) more LHRH released than during control superfusion.

Table 7: Average LHRH release (n=7) (pg/10 min) during the dose response experiment. Means ± Standard Error

MEAN	GABA		MUSCIMOL
Control	13.7±2.8		2.92±0.52
10^{-6}M dose	11.2±1.8**	10^{-7}M dose	3.19±0.83**
10^{-5}M dose	7.65±1.1*	10^{-6}M dose	2.34±0.47
10^{-4}M dose	7.67±1.2*	10^{-5}M dose	2.01±0.67*
Control	6.79±0.8		2.47±0.58

Significant difference ($p<.05$) ** versus *. Paired T-test for percent of control GABA data gives t=2.52 for 10^{-6}M vs 10^{-4}M and t=3.43 for 10^{-6}M vs 10^{-5}M, with six degrees of freedom. Paired T-test for percent of control muscimol data gives t=6.24 for 10^{-7}M vs 10^{-5}M, with six degrees of freedom.

Figure 11: LHRH release during the GABA dose response experiment. The mean level of LHRH released during each ten minute period is shown for the entire experiment. The release during the 10^{-4}M and 10^{-5}M doses of GABA was significantly lower than during the 10^{-6}M dose. Seven rats were used in this experiment.

FIG. 11

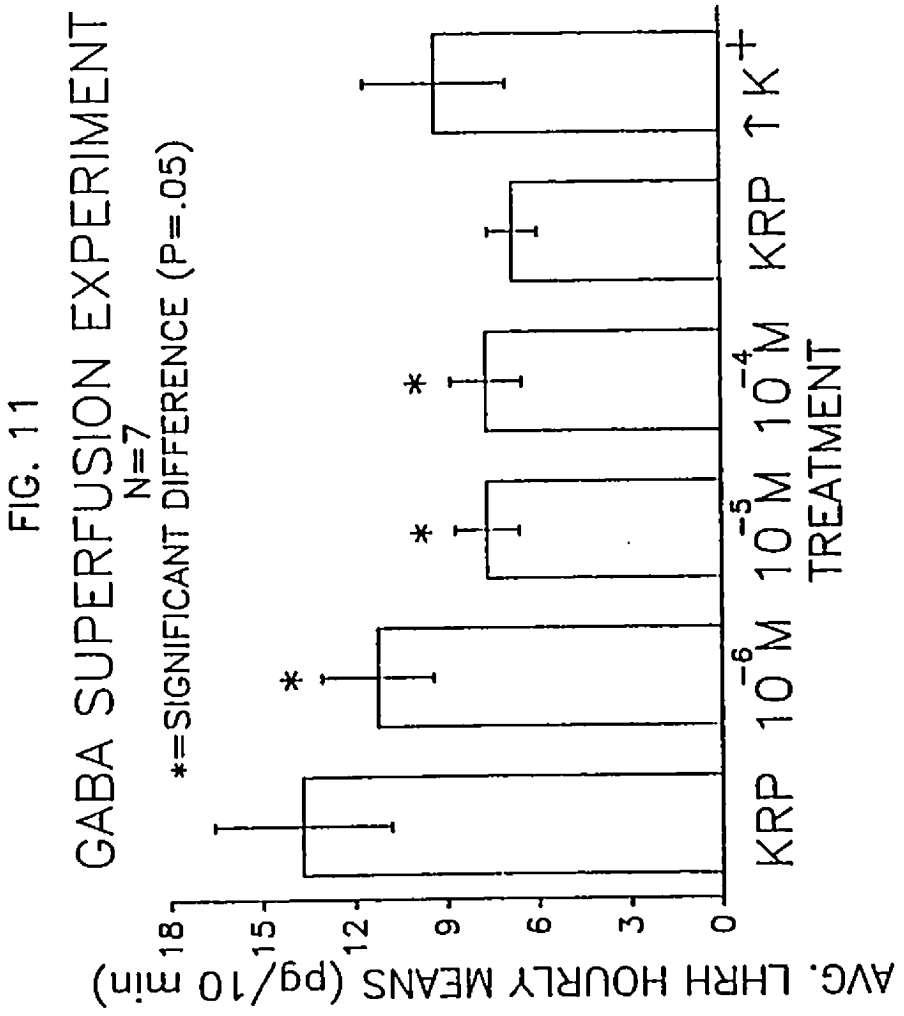
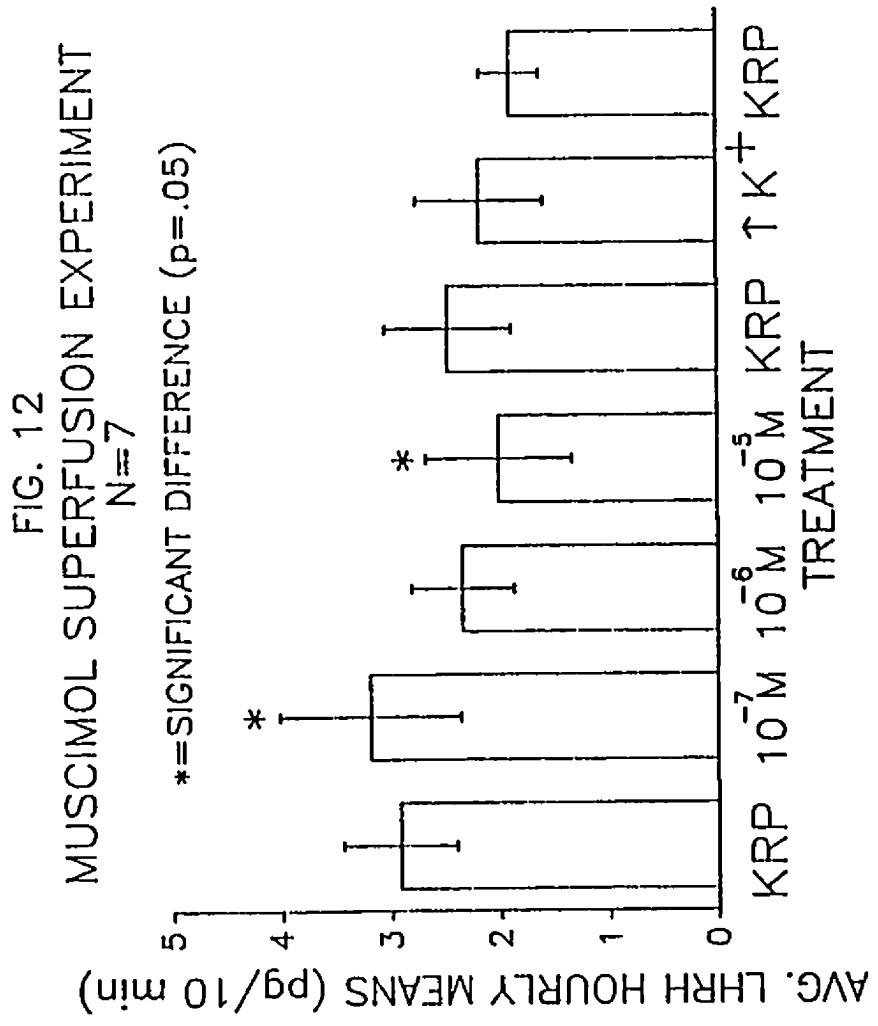


Figure 12: LHRH release during the muscimol dose response experiment. The mean level of LHRH released during each ten minute period is shown for the entire experiment. The release during the 10^{-5}M dose of muscimol was significantly lower than the release during the 10^{-7}M dose. Seven rats were used in this experiment.



4) Receptor subtype:

All of these experiments used $OVXE_2$ rats. All drugs used in these experiments were given in 10^{-4}M doses. The data obtained in these experiments was the amount of LHRH released, as well as the amount of 5-HIAA in every third sample. No significant differences in LHRH release were seen during drug treatment in these experiments. Four rats were used to determine the effect of bicuculine on LHRH release (fig. 13). Seven rats were used to determine the effect of the GABA-B receptor agonist and antagonist (figs. 14 and 15). Eight rats were used to determine the effects of each of the drugs on 5-HIAA release (figs. 16-18). No significant differences in 5-HIAA release were seen during drug treatment in these experiments.

Table 8: Average LHRH release (n=4 for bicuculine, n=7 for both baclofen and the histidine ligand) (pg/10 min) during the receptor subtype superfusion experiment. Boldface indicates the drug (0.1 mM) was being superfused. Means ± Standard Error

MEAN	BICUCULINE	BACLOFEN	HIS. LIG.
First hour	1.4±0.5	0.7±0.2	1.2±0.1
Second hour	1.1±0.4	0.6±0.2	1.1±0.2
Third hour	**1.3±0.6**	**0.7±0.3**	**0.8±0.1**
Fourth hour	**1.8±1.0**	**0.7±0.2**	**1.0±0.2**
Overall	1.4±0.3	0.7±0.03	1.0±0.09

Two way ANOVA for drug treatment and differences between rats. The LHRH release was only significantly ($p<.05$) changed by the histidine ligand treatment. The histidine ligand had $F_{1,27}=6.28$ for drug treatment. However, in each treatment group there were significant differences ($p<.05$) between rats. Bicuculine had $F_{3,15}=22.61$ for between rat differences and Duncan's test showed that the second rat was significantly different ($p<.05$) from the other three rats. Baclofen had $F_{6,27}=20.65$ for between rat differences and Duncan's test showed that the second and fifth rats were significantly different ($p<.05$) from the other five rats. The histidine ligand had $F_{6,27}=8.72$ for between rat differences and Duncan's test showed that the second, third, and fourth rats were signifcantly different ($p<.05$) from the other four.

Figure 13: LHRH release during the $GABA_A$ receptor blockade experiment. The mean level of LHRH released during each ten minute period is shown for the entire experiment. No significant difference in LHRH release was seen during $GABA_A$ receptor blockade. Four rats were used in this experiment.

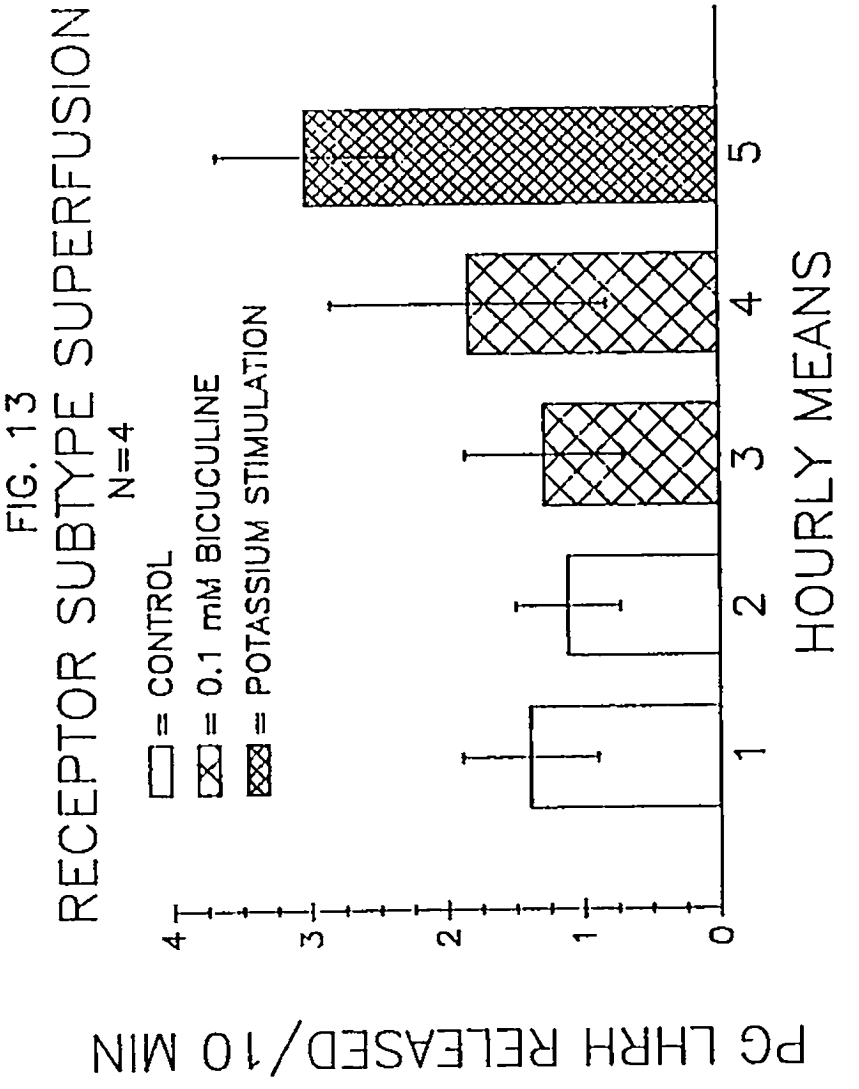


Figure 14: LHRH release during the $GABA_B$ receptor stimulation experiment. The mean level of LHRH released during each ten minute period is shown for the entire experiment. No significant difference in LHRH release was seen during $GABA_B$ receptor stimulation. Seven rats were used in this experiment.

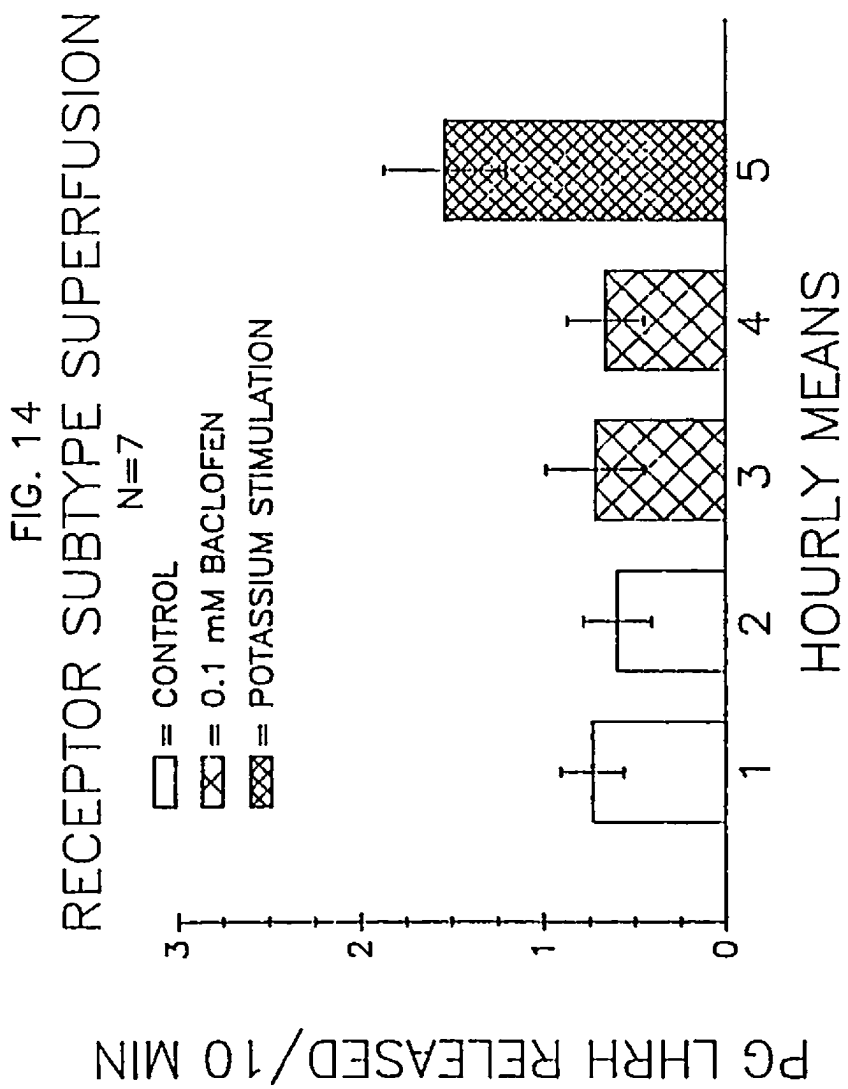


Figure 15: LHRH release during the $GABA_B$ receptor blockade experiment. The mean level of LHRH released during each ten minute period is shown for the entire experiment. No significant difference in LHRH release was seen during $GABA_B$ receptor blockade. Seven rats were used in this experiment.

FIG. 15

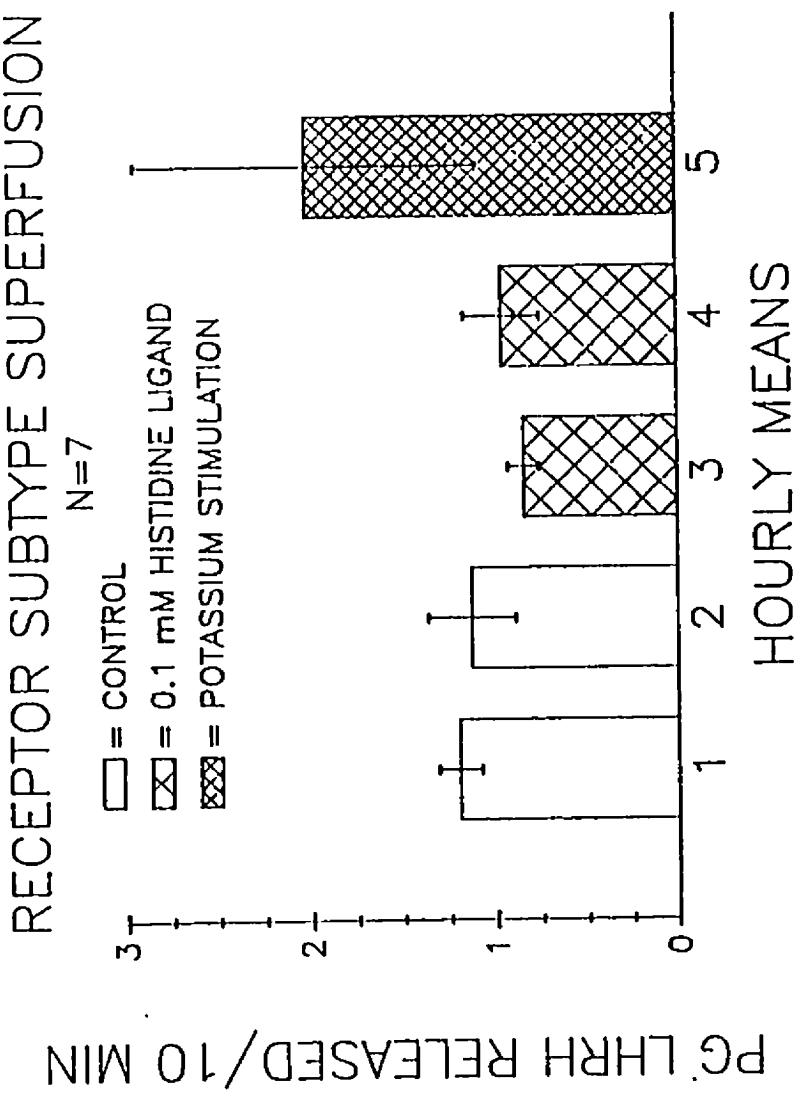


Figure 16: 5-HIAA release during the $GABA_A$ receptor blockade experiment. The amount of 5-HIAA in every third sample is shown for the entire experiment. No significant differences in the amount of 5-HIAA released were seen during $GABA_A$ receptor blockade. Eight rats were used in this experiment.

FIG. 16

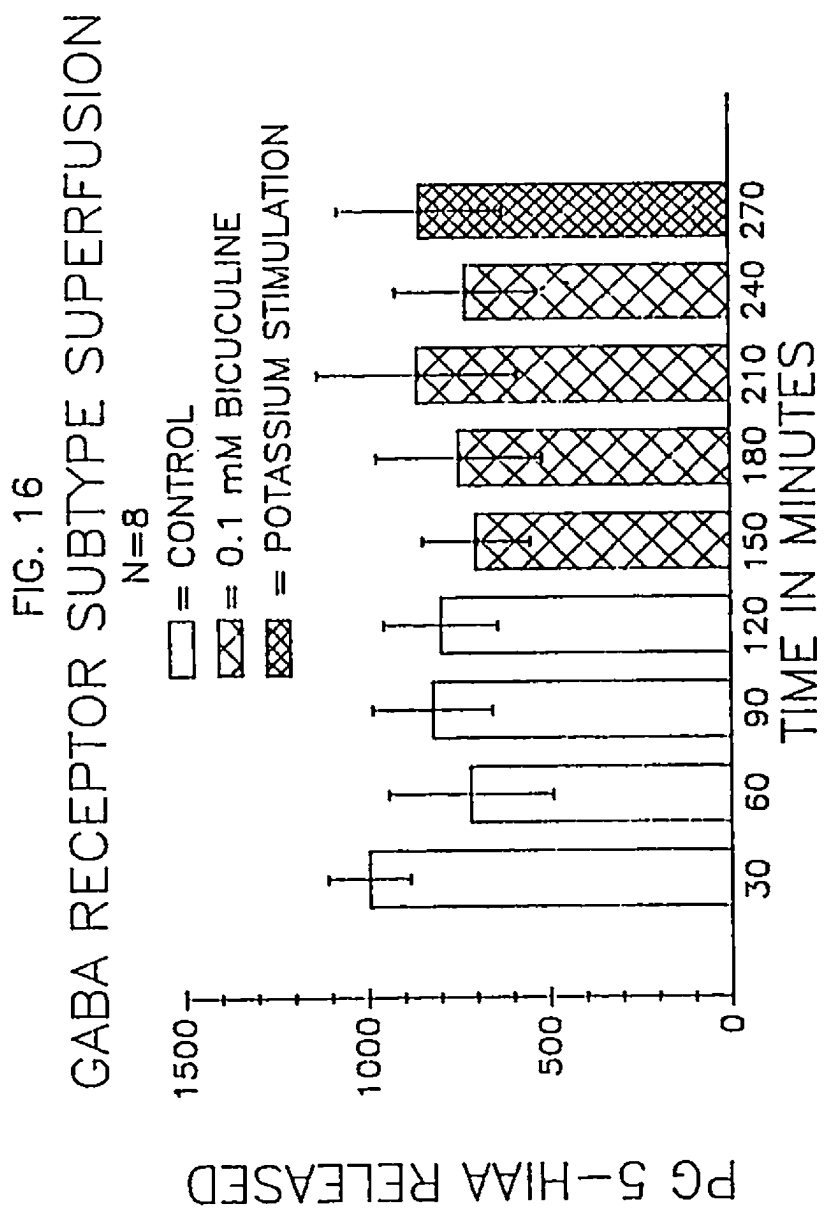


Figure 17: 5-HIAA release during the $GABA_B$ receptor stimulation experiment. The amount of 5-HIAA in every third sample is shown for the entire experiment. No significant difference in the amount of 5-HIAA released was seen during $GABA_B$ receptor stimulation. Eight rats were used in this experiment.

FIG. 17

GABA RECEPTOR SUBTYPE SUPERFUSION

N=8

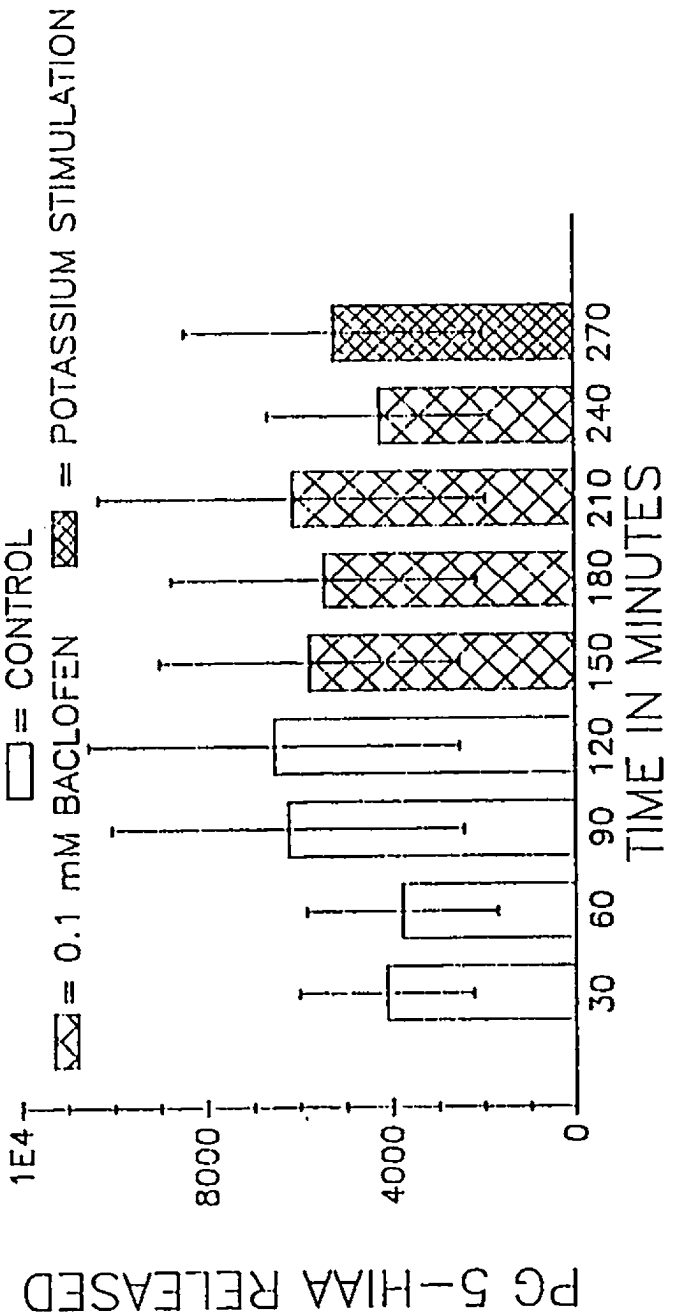


Figure 18: 5-HIAA release during the $GABA_B$ receptor blockade experiment. Eight rats were used in this experiment. The amount of 5-HIAA in every third sample is shown for the entire experiment. No significant differences in the amount of 5-HIAA released were seen during $GABA_B$ receptor stimulation. Eight rats were used in this experiment.

FIG. 18

GABA RECEPTOR SUBTYPE SUPERFUSION

N=8

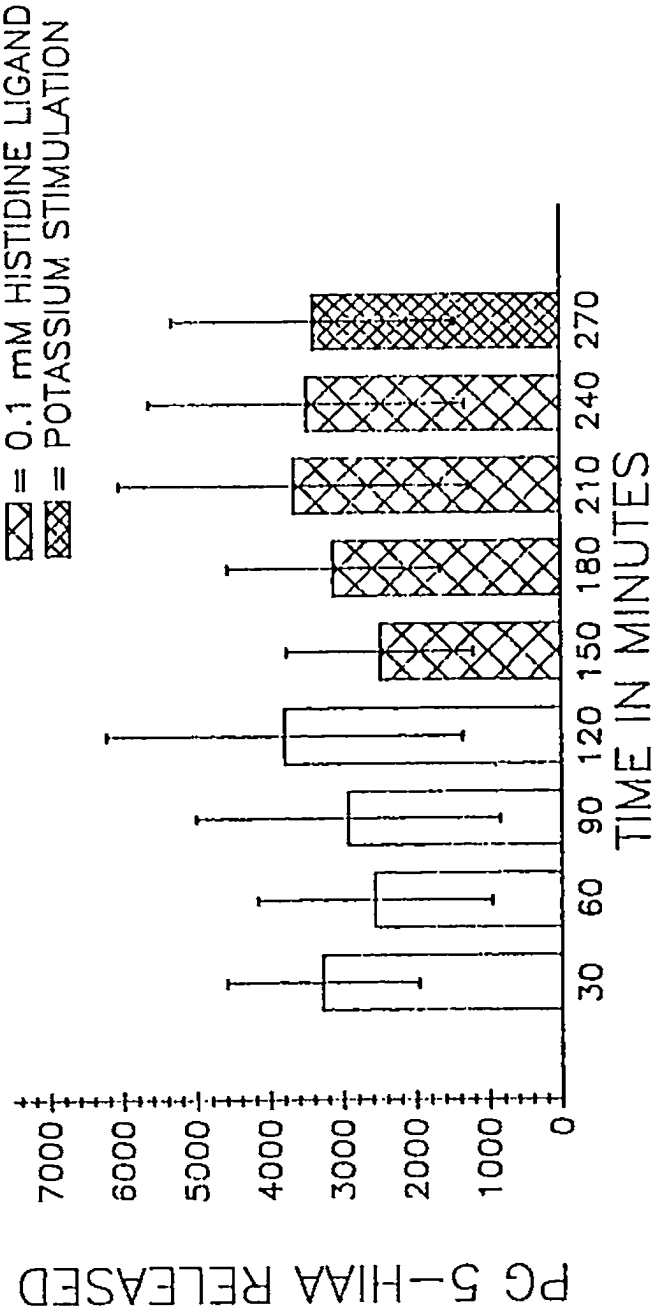


Table 9: Average 5-HIAA release (n=8) (pg) in every third sample during the receptor subtype superfusion experiment. Boldface indicates that drug (0.1 mM) was being superfused. Means ± Standard Error

SAMPLE	BICUCULINE	BACLOFEN	HIS. LIG.
3	995±113	4120±1890	3280±1320
6	715±226	3780±2070	2570±1600
9	820±166	6240±3810	2940±2070
12	**796±157**	**6530±4000**	**3800±2440**
15	**698±147**	**5770±3230**	**2490±1280**
18	**745±228**	**5450±3290**	**3130±1460**
21	**858±274**	**6120±4180**	**3660±2390**
24	858±274	4250±2410	3480±2160
27	849±227	5220±3230	3390±1930
Overall	815±30	5280±336	3193±152

Two way ANOVA for drug treatment and differences between rats. The 5-HIAA release was not changed by any of the drug treatments. Bicuculine had $F_{7,62}=22.72$ for between rat differences and Duncan's test showed that the second and sixth rats were significantly different ($p<.05$) from all others as well as each other, the third rat was significantly different ($p<.05$) from the fourth, fifth, seventh, and eighth, and the eighth rat was significantly different ($p<.05$) from the first and fourth rats. Baclofen had $F_{7,62}=55.53$ for between rat differences and Duncan's test showed that the fourth and eighth rats were significantly different ($p<.05$) from all others as well as each other. The histidine ligand had $F_{7,62}=61.54$ for between rat differences and Duncan's test showed that the second and sixth rats were significantly different ($p<.05$) from all others as well as each other.

B) IN-VIVO PUSH-PULL PERFUSION EXPERIMENTS:

All animals used in the push-pull perfusion experiments were ovariectomized and had estradiol capsules implanted at least 48 hours before the experiment. A total of seventeen animals had cannulas implanted into the median eminence for the push-pull perfusion experiments. The samples obtained in all of the experiments were subjected to HPLC to analyze for indole levels, as well as RIA to analyze for LHRH levels.

1) Control:

Five animals were used in the control experiments. Three rats were perfused for eight hours, one rat was perfused for four hours, and one rat was perfused for four hours and fifty minutes. The perfusion buffer was artificial CSF (CSF) during the entire length of all control experiments. Unfortunately, serotonin remained

undetectable during all control push-pull perfusion experiments. No significant differences were seen when the LHRH release was compared between the different hours of perfusion (fig. 19). However, when the LHRH release data is examined over the course of the experiment its pulsatile nature is evident (fig. 20).

Table 10: LHRH release (six ten minute values [pg] collapsed over each hour) during control push-pull perfusion experiments. Mean ± Standard Error. LHRH pulse period is shown in minutes.

HOUR	RAT#4	RAT#6	RAT#12	RAT#15	RAT#18
One	1.5±0.6	2.3±0.7	1.1±0.3	5.1±1.9	8.7±1.7
Two	0.7±0.2	1.1±0.4	0.64±0	3.1±0.6	10.7±2.5
Three	0.8±0.2	0.64±0	0.9±0.2	3.6±0.4	8.0±6.8
Four	0.8±0.2	1.0±0.3	1.8±0.5	6.2±1.5	1.1±0.2
Five	-	1.4±0.8	1.8±0.8	5.5±0.9	0.7±0
Six	-	0.64±0	0.8±0.1	4.4±0.9	-
Seven	-	2.2±1.5	2.1±0.6	4.7±0.9	-
Eight	-	0.64±0	1.0±0.2	2.6±0.3	-
Mean	0.8±0.3	1.2±0.2	1.3±0.2	4.4±0.4	5.8±2
Period	40	90	48	36	47

One way ANOVA for hour of experiment showed no significant differences in any of the rats.

Four of the control rats had measurable levels of 5-HIAA in their perfusate. None of these rats had significantly different levels of 5-HIAA released during different hours of the experiment. However, the general pattern of release was similar (fig. 21). The release during the first hour was higher and very variable, while during the rest of the experiment lower more constant levels of 5-HIAA release were found.

2) Vehicle:

One rat was used for a vehicle control. The muscimol and GABA were dissolved in 0.1% ethylene glycol for the in-vivo experiments. A four hour perfusion with CSF was followed by a four hour perfusion with CSF containing 0.1% ethylene glycol. No significant difference was seen between hours of the experiment when the

Figure 19: LHRH release during the push-pull perfusion of the median eminence of rat #15. The amount of LHRH released during each ten minute period is shown collapsed over each hour of the experiment. No significant differences in the level of LHRH release were seen between any of the hours of the experiment.

FIG. 19

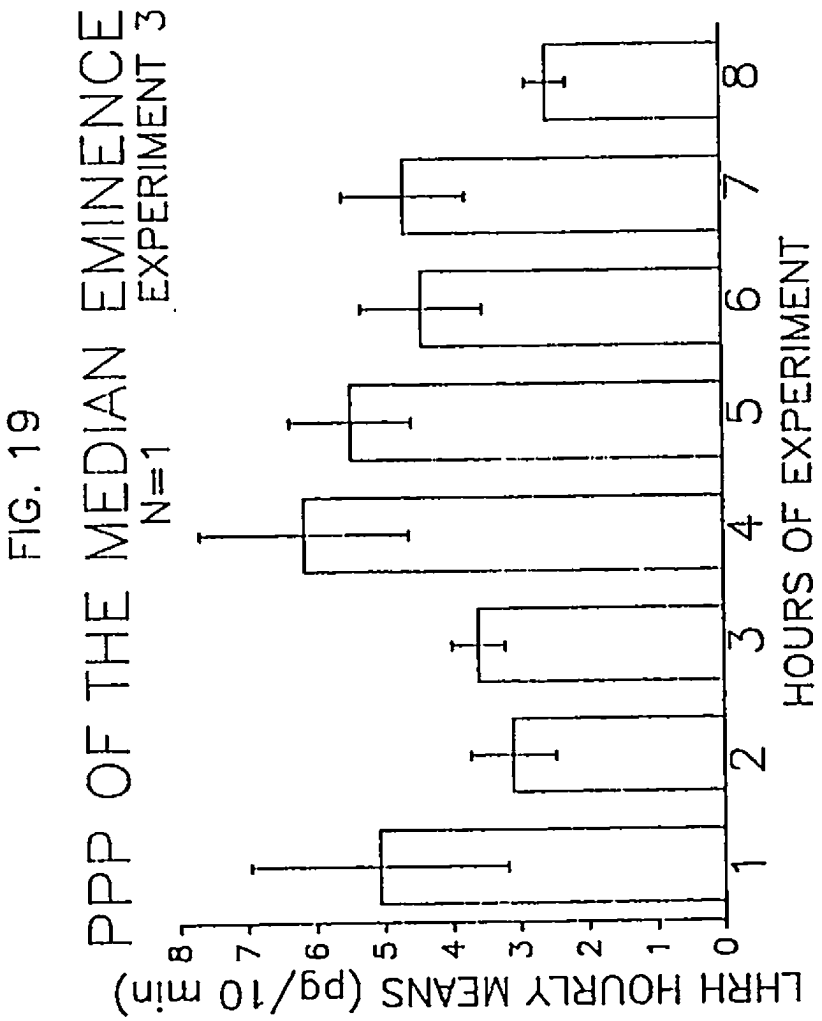


Figure 20: LHRH release during the push-pull perfusion of the median eminence of rat #15. The amount of LHRH released is shown for the entire experiment. The D indicates the onset of the rat's normal dark cycle, while the asterisks indicate peaks identified by PULSAR.

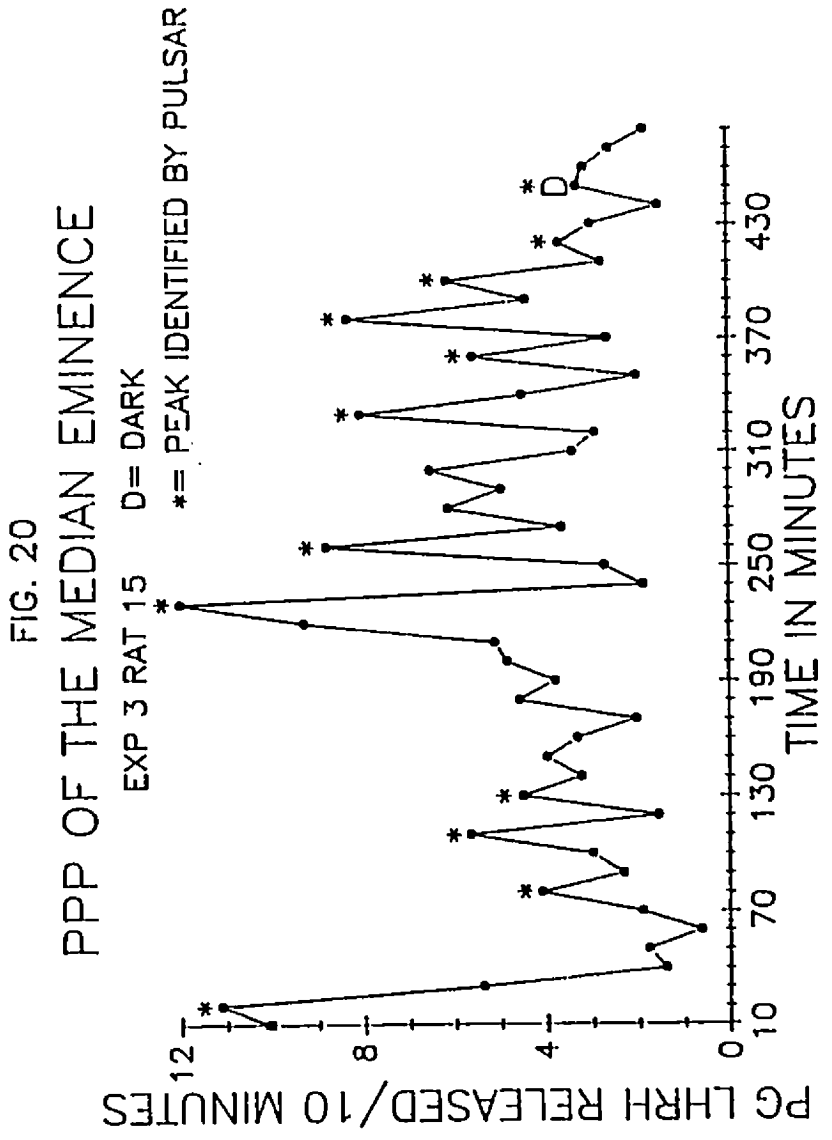
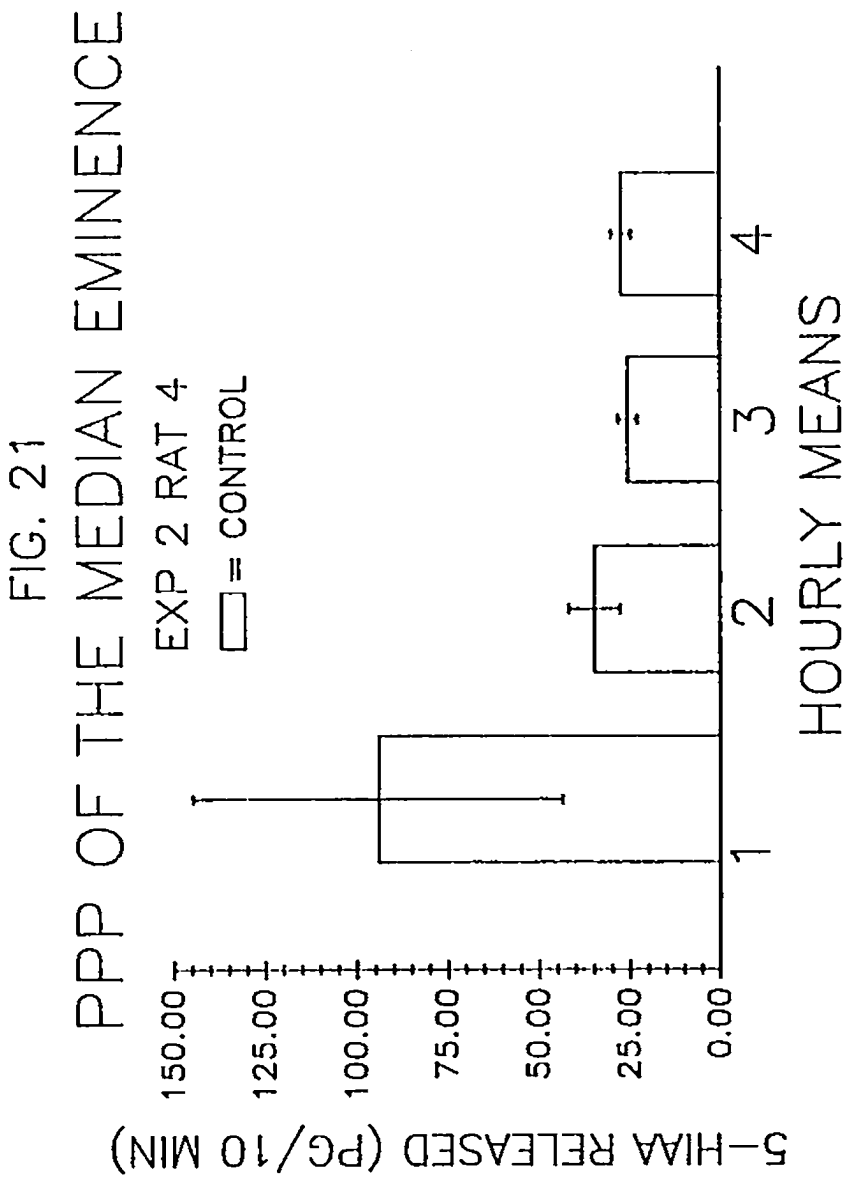


Figure 21: 5-HIAA release during the push-pull perfusion of the median eminence of rat #4. The level of 5-HIAA released during each ten minute period is shown collapsed over each hour of the experiment. No significant differences in the level of 5-HIAA release were seen between any of the hours of the experiment.



LHRH data was analyzed by ANOVA (fig. 22). However, when an ANOVA was run on the 5-HIAA data the second through sixth hours were significantly greater than the first hour and the last two hours (fig. 23).

Table 11: The vehicle push-pull perfusion experiment LHRH and 5-HIAA release (six ten minute values [pg] collapsed over each hour). Boldface indicates that vehicle (0.1% ethylene glycol) was perfused.

HOUR	LHRH	5-HIAA
One	1.2±0.27	80.9±22
Two	0.97±0.25	152±3.5
Three	1.7±0.47	206±28
Four	2.1±0.58	292±8.0
Five	**1.4±0.18**	**208±11**
Six	**1.3±0.13**	**174±23**
Seven	**1.6±0.27**	**102±9.0**
Eight	**1.6±0.21**	**97.3±2.7**
Mean	1.5±0.12	164±25

One way ANOVA for hourly differences done on the 5-HIAA data $F_{7,39}=17.59$. Duncan's test showed that the fourth hour was significantly different ($p<.05$) from all others, and that the first, seventh and eighth hours were significantly different ($p<.05$) from all others, and the second hour was significantly different ($p<.05$) from the third and fifth. These differences are not related to vehicle perfusion.

3) GABA:

Five rats were used in the GABA experiments. A four hour perfusion with CSF was followed by a four hour perfusion with 10^{-6}M GABA dissolved in CSF containing 0.1% ethylene glycol. All of the animals had LHRH in their perfusates. An ANOVA run on the LHRH data showed some significant differences between hours. However, these differences did not correspond to drug treatments (figs. 24 and 25).

Figure 22: LHRH release during the vehicle push-pull perfusion experiment. The level of LHRH released during each ten minute period is shown collapsed over each hour of the experiment. No significant differences in the level of LHRH release were seen between any of the hours of the experiment.

FIG. 22

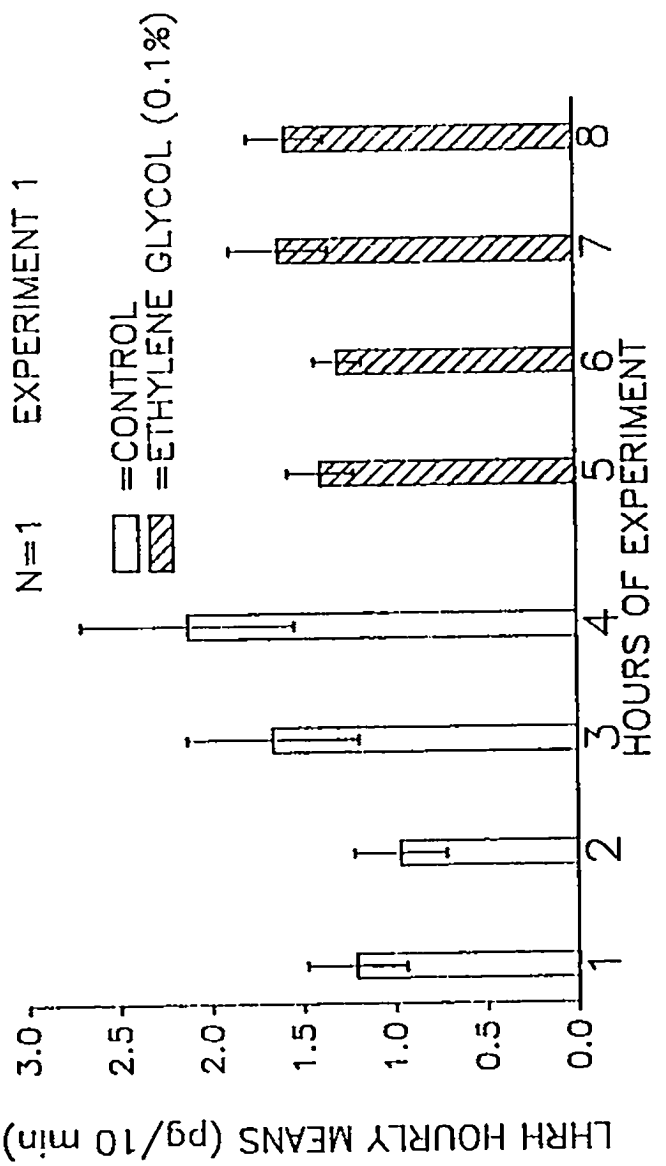


Figure 23: 5-HIAA release during the vehicle push-pull perfusion experiment. The level of 5-HIAA released during each ten minute period is shown collapsed over each hour of the experiment. A significant increase in 5-HIAA release is seen during the second through sixth hours of the experiment. This increase occurred during both control and vehicle perfusion.

FIG. 23

PPP OF THE MEDIAN EMINENCE

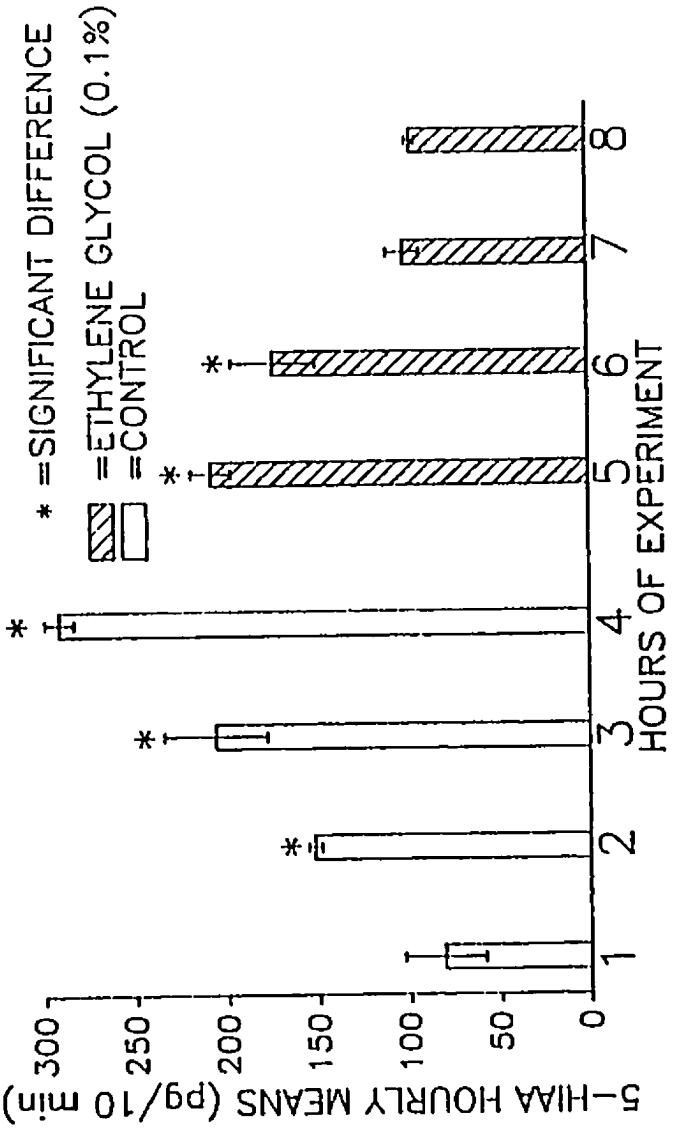


Figure 24: LHRH release during a GABA stimulation push-pull perfusion experiment. The level of LHRH released during each ten minute period is shown collapsed over each hour of the experiment. The third, seventh, and eighth hours had a significantly lower level of LHRH release.

FIG. 24

PPP OF THE MEDIAN EMINENCE

N=1 EXPERIMENT 1

* =SIGNIFICANT DIFFERENCE (p=.05)

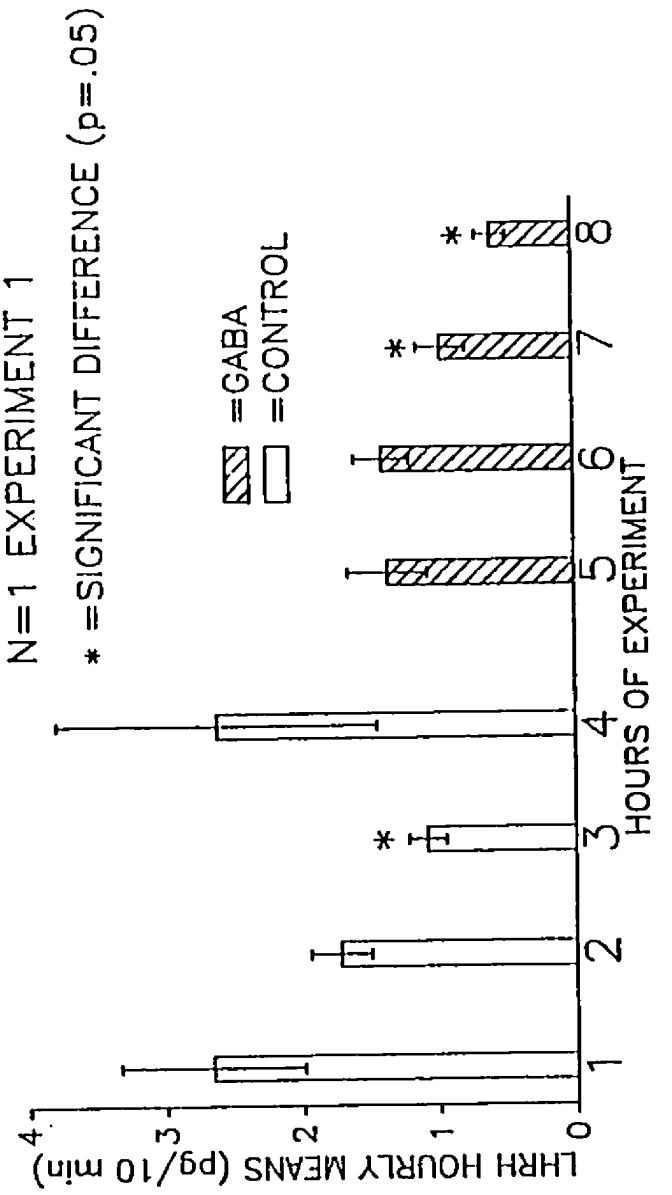


Figure 25: LHRH release during a GABA stimulation push-pull perfusion experiment. The level of LHRH released during each ten minute period is shown collapsed over each hour of the experiment. The fifth and sixth hours had a significantly higher level of LHRH release.

FIG. 25

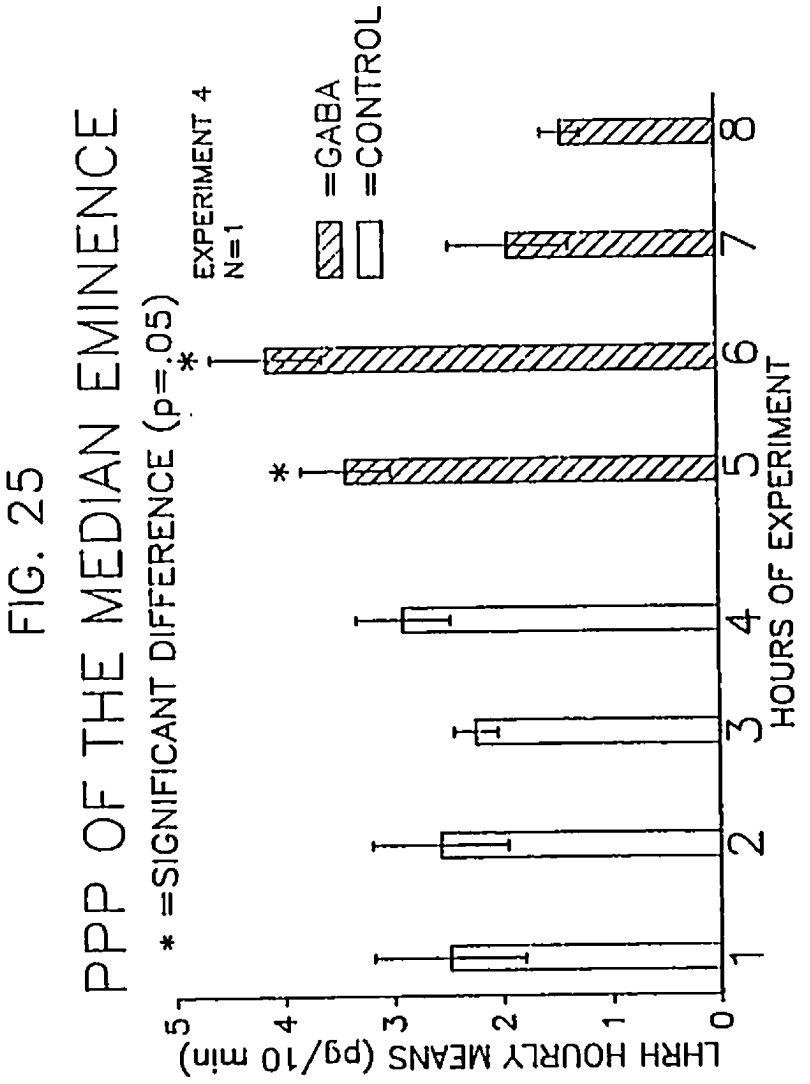


Table 12: GABA push-pull perfusion experiments LHRH release (six ten minute values [pg] collapsed over each hour). Boldface indicates GABA (10^{-6}M) was perfused. Mean ± Standard Error. LHRH pulse period is given in minutes.

HOUR	RAT#8	RAT#13	RAT#14	RAT#16	RAT#20
One	2.7±0.67	0.9±0.12	1.1±0.23	1.5±0.16	2.5±0.69
Two	1.7±0.22	0.6±0.12	1.1±0.33	2.8±1.12	2.6±0.62
Three	1.1±0.14	1.5±0.3	1.4±0.44	1.9±0.43	2.2±0.2
Four	2.6±1.17	0.8±0.16	2.1±0.78	3.8±1.26	2.9±0.43
Mean	2.0±0.38	1.0±0.19	1.4±0.24	2.5±0.51	2.6±0.14
Period	30	48	34	40 34	
Overall mean=1.9±0.31		Period mean=37±3			
Five	**1.4±0.3**	**0.8±0.22**	**1.0±0.46**	**2.2±0.77**	**3.4±0.41**
Six	**1.4±0.2**	**1.0±0.29**	**2.0±0.85**	**2.2±0.36**	**4.1±0.51**
Seven	**1.0±0.18**	**0.7±0.15**	**0.7±0.14**	**1.9±0.17**	**1.9±0.55**
Eight	**0.6±0.11**	**1.1±0.28**	**0.9±0.17**	**1.8±0.48**	**1.4±0.18**
Mean	**1.1±0.19**	**0.9±0.09**	**1.2±0.29**	**2.0±0.1**	**2.7±0.63**
Period	**40**	**48**	**30**	**30 34**	
Overall mean=1.6±0.38		**Period mean=36±3**			

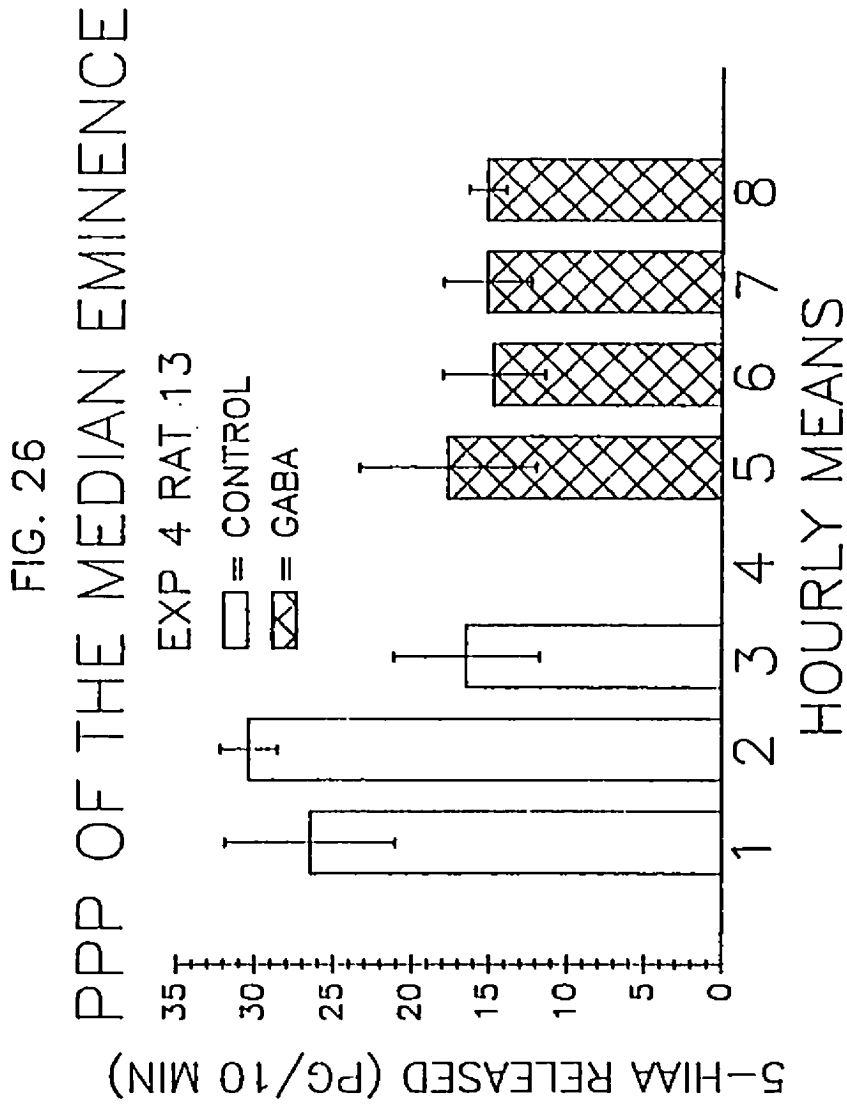
One way ANOVA for hourly differences done on rats' number eight and twenty, $F_{7,40}=2.79$ for rat eight, and $F_{7,40}=3.33$ for rat twenty. Duncan's test showed that for rat eight the third, seventh, and eighth hours were significantly different ($p<.05$) from the first and fourth hours, while for rat twenty the sixth hour was significantly different ($p<.05$) from all except the fifth hour, and the eighth hour was significantly different ($p<.05$) from the fifth and sixth hours.

Only three rats had detectable levels of 5-HIAA released during the GABA superfusion experiments. The release that was seen was intermittent. The only significant differences between hours were seen between hours without 5-HIAA release and hours during which 5-HIAA release occurred. These hours did not correspond to drug treatment and control perfusion (fig. 26).

4) Muscimol: ($GABA_A$ receptor agonist)

Six rats were used in the muscimol experiments. The first half of each experiment was a control period with just CSF perfused, and 10^{-6}M muscimol dissolved in CSF containing 0.1% ethylene glycol was perfused during the second half of the experiment. All of the eight hour experiments had both LHRH and 5-HIAA

Figure 26: 5-HIAA release during a GABA stimulation push-pull perfusion experiment. The level of 5-HIAA released during each ten minute period is shown collapsed over each hour of the experiment. The only significant difference in 5-HIAA release between hours of the experiment is during the fourth hour when no 5-HIAA release occurred.



data, while the six hour experiment only had LHRH data. None of the LHRH data showed any significance when an ANOVA was run (fig. 27). However, two of the rats with 5-HIAA data did have a significant difference between hours of the experiment. Unfortunately, these two rats did not differ in the same manner. In one experiment muscimol decreased the 5-HIAA release (fig. 28), while in the other experiment it increased 5-HIAA release (fig. 29).

Table 13: Muscimol push-pull perfusion experiments LHRH release (six ten minute values [pg] collapsed over each hour). Boldface indicates muscimol (10^{-6}M) was perfused. LHRH pulse period is given in minutes.

HOUR	RAT# 3	RAT# 5	RAT# 6	RAT#21	RAT#23	RAT#26
One	1.1±0.1	1.6±0.3	1.0±0.2	0.8±0.1	0.7±0	0.7±0.1
Two	1.1±0.3	1.1±0.1	1.0±0.2	3.7±2.9	0.7±0.1	4.7±2.3
Three	1.4±0.2	0.9±0.3	0.9±0.3	0.6±0	2.0±1.2	1.3±0.6
Four	0.6±0.1	1.1±0.2	1.1±0.3	1.7±0.8	0.6±0	0.6±0
Mean	1.1±0.2	1.2±0.2	1.0±0	1.7±0.7	1.0±0.3	1.8±1.0
Period	34	48	40	60	120	80
Overall mean=1.3±0.1			Period mean=63.7±13.1			
Five	**0.8±0.3**	**0.8±0.3**	**1.2±0.3**	**0.7±0.5**	**0.9±0.2**	**12±11**
Six	**0.9±0.2**	**0.9±0.2**	**3.9±3**	**0.7±0.1**	**0.7±0**	**1.2±0.3**
Seven	**0.5±0.2**	**-**	**1.8±0.6**	**0.6±0**	**0.7±0.2**	**1.3±0.6**
Eight	**0.8±0.3**	**-**	**1.4±0.2**	**1.2±0.6**	**0.7±0.2**	**3.4±3**
Mean	**0.8±0.1**	**0.9±0.1**	**2.1±0.6**	**0.8±0.1**	**0.8±0.1**	**4.5±2.6**
Period	**34**	**40**	**34**	**80**	**80**	**40**
Overall mean=1.7±0.6			**Period mean=51.3±9.1**			

One way ANOVA for hourly differences was not significant for any rats.

Figure 27: LHRH release during a $GABA_A$ receptor stimulation push-pull perfusion experiment. The level of LHRH released during each ten minute period is shown collapsed over each hour of the experiment. There were no significant differences in the amount of LHRH released during different hours of the experiment.

FIG. 27
PPP OF THE MEDIAN EMINENCE

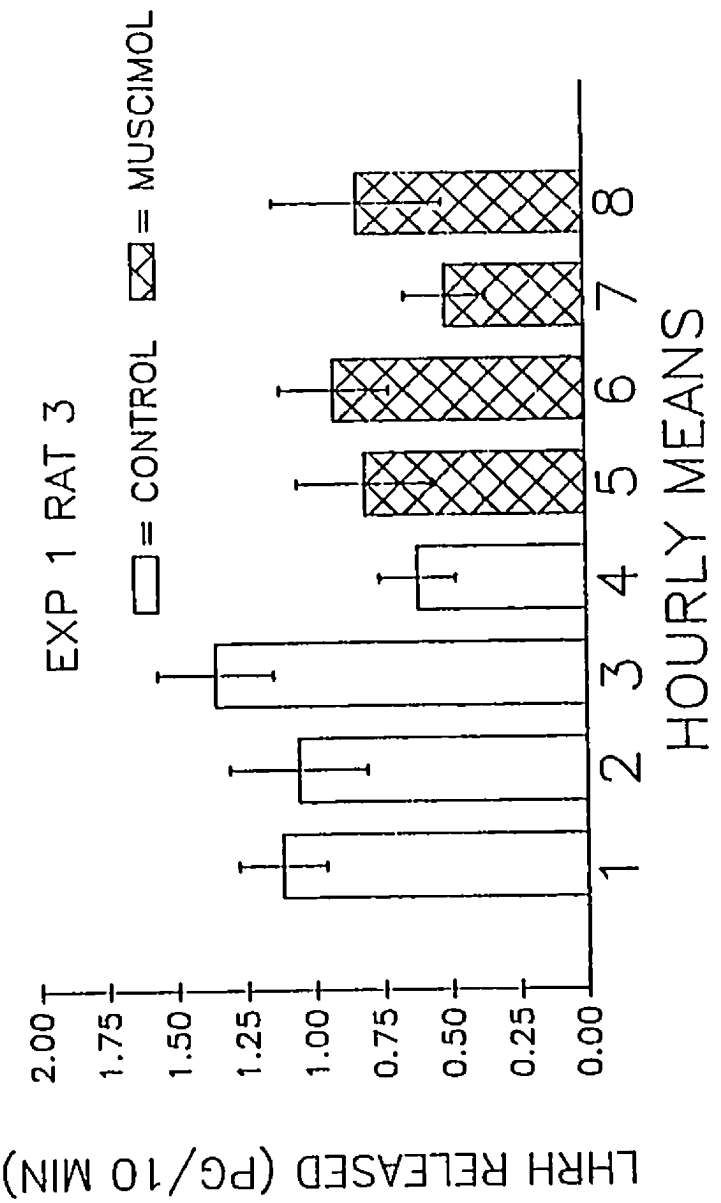


Figure 28: 5-HIAA release during a $GABA_A$ receptor stimulation push-pull perfusion experiment. The level of 5-HIAA release during each ten minute period is shown collapsed over each hour of the experiment. The level of 5-HIAA release during the last three hours was significantly lower than it was during the first five hours.

FIG. 28

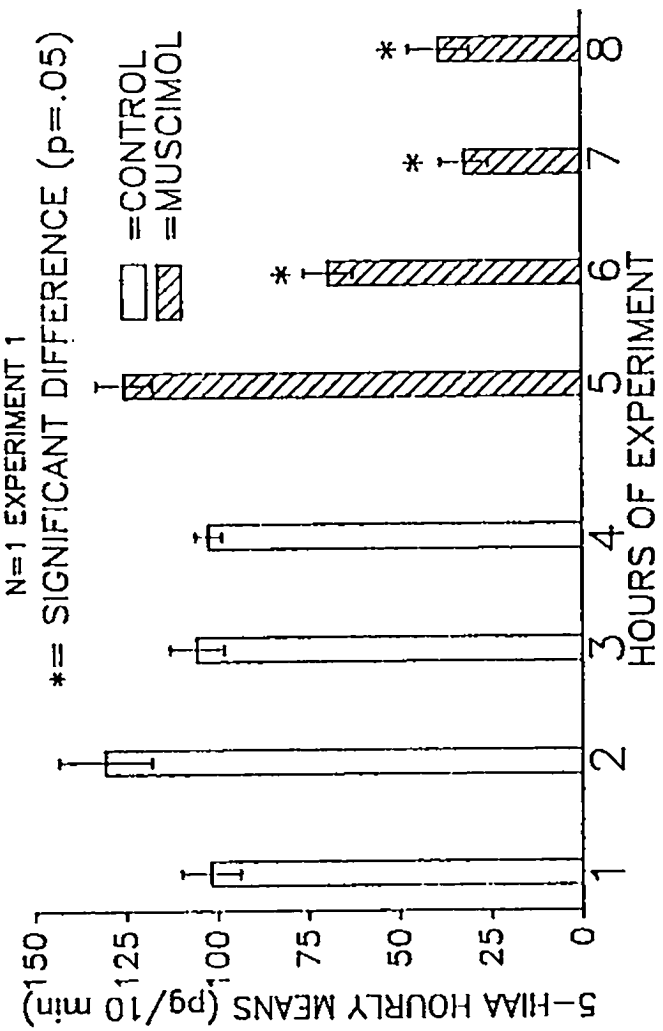
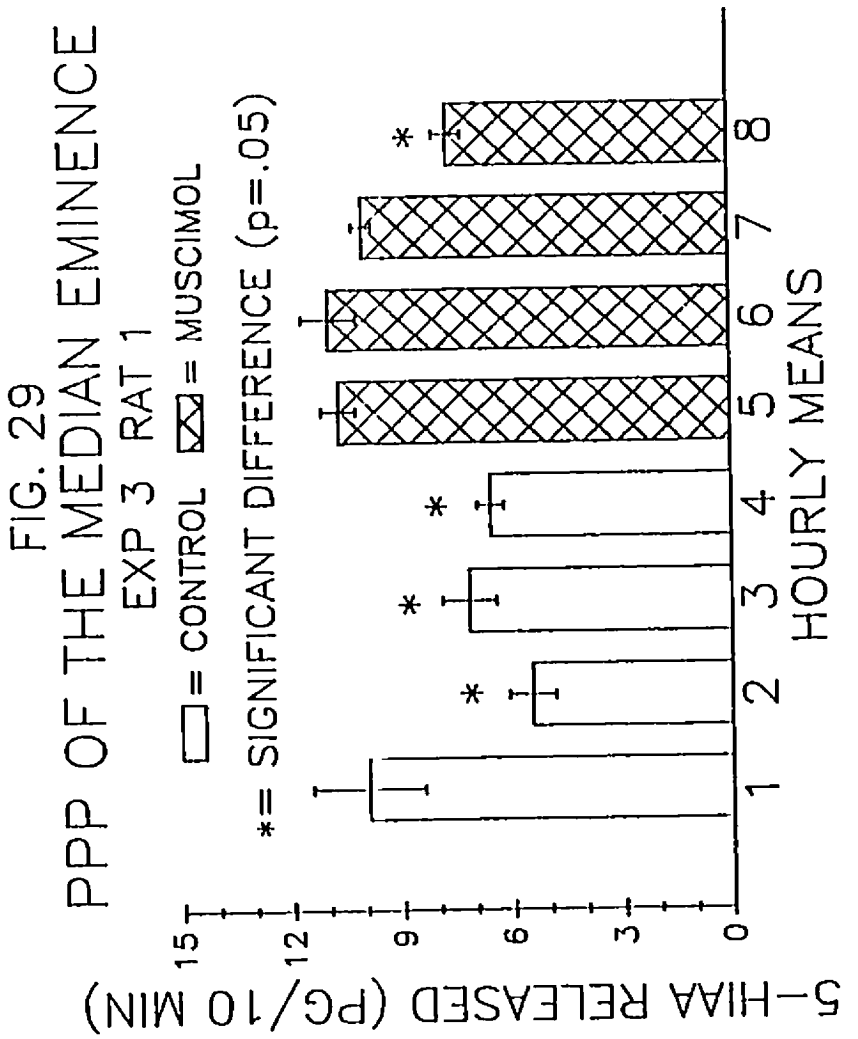


Figure 29: 5-HIAA release during a $GABA_A$ receptor stimulation push-pull perfusion experiment. The level of 5-HIAA release during each ten minute period was collapsed over each hour of the experiment. The level of 5-HIAA release during the second, third, forth, and eighth hours was significantly lower than the rest of the experiment.



DISCUSSION

EFFECT OF ESTRADIOL:

The modulation of the hypothalamic LHRH pulse generator by GABA is complex and regulated by the integration of the brain and ovaries. Estradiol and progesterone released by the ovaries and adrenal glands transmit the regulatory signal to the hypothalamus. In female mammals, the ovarian steroid hormone, estradiol, is released in a cyclic manner. It increases during the time that the follicle is maturing, reaching a peak immediately prior to ovulation. Following ovulation plasma estradiol levels decrease and remain relatively low until another follicle begins to mature. These fluctuations in estradiol levels cause a change in the effect of GABA on LHRH release from the hypothalamus (figures 9 and 10).

GABA is an inhibitory neurotransmitter that can decrease the ability of a neuron to fire (by opening chloride channels), as well as the ability of the firing axon to release neurotransmitter (by closing calcium channels). Estrogen receptive neurons, in the medial preoptic/anterior hypothalamic area, that contain glutamic acid decarboxylase (the enzyme necessary for synthesizing GABA) have been described (20). Also in the medial preoptic area, glutamic acid decarboxylase containing neurons make synaptic contact with LHRH containing neurons (44). The structural requirements necessary for mediating an action of estradiol on LHRH neurons via GABA secreting neurons are present in the medial preoptic area (figure 1). The system of neurons shown in figure 1 may be responsible for the inhibition of LHRH release caused by GABA stimulation.

When the proper transmitter system (such as norepinephrine) provides enough stimulation a pulse of LHRH is released (29). The LHRH pulse is released at the correct time for the estradiol stimulated pituitary to amplify this signal. Previous LHRH pulses can also prime the pituitary gonadotropes (35). The potentiation of the effect the LHRH pulse has on gonadotropes due to high plasma estradiol levels is maximized during proestrus (70,65,36,15). This potentiation, combined with the

priming of the gonadotropes, results in a surge in plasma LH levels that can trigger ovulation. Although GABA stimulation can decrease LHRH release it may also act to synchronize the hypothalamic LHRH pulse generator. By keeping LHRH neurons slightly inhibited GABAergic systems within the hypothalamus can prepare them for a synchronous firing pattern induced by stimulatory noradrenergic input.

EFFECT OF GABA ON FREQUENCY OF LHRH RELEASE:

The effects of GABA were not manifested in changes in frequency of pulsatile release. All of the PULSAR analyzed data showed no significant difference in the frequency of pulses released when GABA (or a GABA receptor agonist or antagonist) was present in the medium. The changes that occurred under GABA stimulation were reflected in the average amount released during the stimulation. The number of pulses seen during GABA stimulation was not significantly different from the number seen under control conditions in any of the experiments. However, the relative amount of LHRH released during GABA stimulation did change when compared with control conditions in some experiments (figure 10).

The inability of GABA to affect LHRH pulsatility reflects the nature of GABAergic modulation. The literature suggests that activation of GABAergic systems will produce a generalized decrease in the excitability of another neuronal system. GABA appears to have a minor influence that can fine tune the actions of other, more potent, neurotransmitter systems (52). This type of modulation would not significantly change the period of the LHRH pulse generator. However, it would decrease the relative amount of LHRH released.

IN-VITRO EFFECTS OF GABA STIMULATION:

In the ovariectomized rat with a low constant level of estradiol ($OVXE_2$), GABA stimulation decreased LHRH release in-vitro (figure 10). This decrease in LHRH release was not seen when intact proestrus rats (which have a steadily

increasing, relatively high level of estradiol) were stimulated with GABA (figure 9). This could be due to an inability of GABA inhibition to affect LHRH release under conditions of high (and rapidly increasing) plasma estradiol levels. Although GABA release is directly regulated by estrogens (20) the regulation may require the estradiol level to be elevated for a longer period of time. The $OVXE_2$ rats had their estradiol level elevated for two days prior to the experiment.

When OVX rats are treated with fifty micrograms of estradiol subcutaneously, followed two days later by another subcutaneous injection of two and a half milligrams of progesterone, a surge of LH results. If the $GABA_A$ receptor agonist muscimol or the $GABA_B$ receptor agonist baclofen are injected concomitantly with progesterone the LH surge is blocked (2). Conversely, in intact or gonadectomized male rats GABA and $GABA_A$ receptor agonists can stimulate LHRH release from hypothalamic fragments (59). In OVX rats the release of GABA in the medial preoptic area is significantly lower than in $OVXE_2$ rats (16). However, in OVX rhesus monkeys no change in GABA release within the preoptic area is seen during estrogen treatment. In monkeys the medial basal hypothalamus is the location where GABA release changes during estrogen treatment (24).

The rat is the most commonly used experimental model for exploring the neurotransmitter interactions that are pertinent to LHRH release. Several investigators have found GABA to be involved in these interactions. Muscimol, a $GABA_A$ receptor agonist, reduces norepinephrine and dopamine turnover in the preoptic-anterior hypothalamic area of OVX rats. Concurrent with this reduction in neurotransmitter turnover serum LH levels are reduced (25). Baclofen, a $GABA_B$ receptor agonist, can completely suppress the LH increase caused by naloxone (an opiate antagonist) (51). Stimulation of the median raphe nucleus (an area containing serotonin cell bodies) can inhibit LH release on the afternoon of proestrus. This inhibition of LH release during proestrus can be blocked by GABA antagonists (56).

During proestrus, the intrinsic hypothalamic GABA neurons may already be exerting the maximum amount of inhibition possible via GABAergic system activation. In $OVXE_2$ rats administration of $GABA_A$ and $GABA_B$ antagonists can potentiate the stimulatory effect of norepinephrine on LHRH release (29). Similar experiments on proestrus rats would determine the amount of inhibition that the GABAergic system is exerting at that time. Alternatively, another neurotransmitter system may be activated during proestrus that cancels out any effect GABA stimulation may have. The plasma estradiol levels are low prior to proestrus and estrogen receptors in GABA neurons may need a longer period of stimulation before they can be activated.

The inability of GABA activation to decrease LHRH release during proestrus is an interesting phenomenon. How was the effect of GABA stimulation compromised by the low levels of LHRH released in these experiments? In the control experiments, the amount of LHRH released during proestrus was greater than the amount that was released with any other hormonal state (figure 2). The GABA stimulation experiments on proestrus rats had much lower amounts of LHRH released (figure 9). These lower amounts of LHRH release may have been at basal levels. This could have prevented the detection of any further reduction in LHRH release during the GABA superfusion.

Seasonal changes in LHRH release may have contributed to the basal level of LHRH release that was seen during the proestrus GABA experiment. The proestrus control experiment was conducted in the fall, while the proestrus GABA experiment was conducted in the winter, a difference of three months. Another factor that may have influenced LHRH release were the previous cycles before the experiment. The cycles of the rats in the proestrus control experiment were monitored continuously from their time of arrival until they were used in the experiment. The rats in the proestrus GABA experiment had their cyclicity determined twenty days prior to the actual experiment. Although all rats in the experiment were determined to be in proestrus on

the morning of the experiment they may not have been cycling properly before the experiment.

In-vivo push-pull perfusion of the preoptic area in $OVXE_2$ rats indicates that when plasma LH levels were low GABA release was increased (16). The rostral preoptic area of rats contains dispersed LHRH neurons that can interact to allow the orchestration of pulsatile activity (18). Several neurotransmitter systems have terminals within this area and differing degrees of potency in the modulation of LHRH release (10,20,28,44,45). The heterogeneous nature of the preoptic area allows the different neurotransmitter systems to influence the LHRH pulse generator.

The level of GABAergic stimulation needed to change LHRH release was determined in the dose-response superfusion experiments. The effect of GABA in the $OVXE_2$ rat was related to dose (figure 11). A 10^{-6}M dose caused a slight, but not significant, decrease in the amount of LHRH released. When the dose was increased to 10^{-5}M a significant decrease in the amount of LHRH released occurred (when compared with the 10^{-6}M dose). This same significant decrease was seen when the 10^{-4}M dose was compared with the 10^{-6}M dose. The minimum dose of GABA needed to significantly decrease LHRH release in $OVXE_2$ rats is 10^{-5}M; however, a dose of 10^{-4}M is just as effective. The minimum effective dose of GABA must be between 10^{-6}M and 10^{-5}M since the significant decrease in LHRH release occurred between these doses, and the decrease caused by 10^{-4}M was similar to the decrease resulting from 10^{-5}M.

$GABA_A$ receptor stimulation by the agonist muscimol can also decrease LHRH release in $OVXE_2$ rats (figure 12). The 10^{-5}M dose was the dose that caused a significant decrease in LHRH release. However, this decrease was only significant when it was compared to the level of release seen with a 10^{-7}M dose. There was not a significant difference between the 10^{-6}M and 10^{-5}M doses. The receptor subtype

experiments did not have conclusive results. These inconclusive results could be due to the high concentration of drugs used in these experiments.

All drugs used in the receptor subtype determination experiments were administered in a dose of 10^{-4}M. This concentration of GABA was effective in reducing LHRH release in $OVXE_2$ rats (figure 11). However, the specific agonists and antagonists may be activating autoreceptor mechanisms at these concentrations. Lower concentrations of $GABA_A$ receptor agonists and antagonists can stimulate LHRH release in male rats (59). $GABA_B$ receptor stimulation in male rats can suppress the LH surge normally seen during opiate receptor blockade (51). Because of their specific actions, these drugs, at this concentration, may be effectively short circuiting any effect of GABA stimulation.

Another factor in the lack of effect of subtype specific drugs is the lack of reuptake systems and metabolism of these drugs. The high concentrations of these drugs would then be present throughout the stimulation period. This did not appear to affect the viability of the tissue since stimulation with 60 mM potassium increased LHRH release in these experiments (figures 13-15). The specific subtype of GABA receptors responsible for the effect of GABA stimulation may be different in female rats. Both $GABA_A$ and $GABA_B$ receptor antagonists can amplify LH secretion caused by norepinephrine stimulation in $OVXE_2$ rats (29).

No effect was seen on the release of 5-HIAA (an indicator of serotonin metabolism) with any of the receptor subtype agonists or antagonists. The metabolism of serotonin is unaffected by treatment with these receptor subtype specific drugs. None of the GABA stimulation experiments on $OVXE_2$ rats showed changes in the serotonin system caused by GABA. The only change noted during the control experiments was the consistent decrease in 5-HIAA release for both proestrus and $OVXE_2$ hormonal states (figures 6,8). The decreased release of 5-HIAA as the control proestrus and $OVXE_2$ experiments progressed may have been due to a decrease in the

metabolism of serotonin. This could be the result of an expected slow decline in enzyme viability while the tissue was being maintained in-vitro. Detectable levels of serotonin were released intermittently during all of the control experiments. Many proestrus and $OVXE_2$ rats had serotonin released during the later hours of the control experiments yet invariably 5-HIAA levels decreased. This decrease is due to a decreased conversion of serotonin to 5-HIAA, perhaps regulatory signals for serotonin release and metabolism were cut off when the tissue was removed from the brain, since serotonin is released intermittently throughout the experiment. The amount of LHRH released throughout all of the control experiments was also relatively constant, with no significant changes.

As expected, proestrus rats released the most LHRH, followed by estrus rats, with the least amount being released by OVX and $OVXE_2$ rats (figures 2-4). The proestrus rats should release the greatest amount of LHRH because this hormonal state is associated with the LH peak that triggers ovulation. In estrus rats the pituitary LHRH levels are lower and relatively constant (63), and this was reflected in the significantly lower amounts of LHRH released by the hypothalamus (figure 3). There was no significant difference between the amount of LHRH released by the OVX or $OVXE_2$ rats (figure 4). However, the amount of LHRH released was much lower in these two hormonal states than it was during any of the experiments using intact rats (estrus or proestrus experiments). The ovariectomized rats had a lower level of LHRH release than the intact rats, possibly due to a negative feedback effect of much lower levels of estradiol in this system.

The exposure of the tissue to estradiol affected the amount of serotonin that was released. The duration of exposure to estradiol appeared to be the regulatory factor influencing serotonin release. The two states which had a longer period of exposure to estradiol (estrus and $OVXE_2$) had a higher level of serotonin release (figure 7). If estradiol was absent (OVX), or had been present for just a short time (proestrus), a

lower level of serotonin release was seen (figure 9). Previous studies in our laboratory showed that serotonin could significantly increase the period of LHRH pulses in OVX rats while in $OVXE_2$ rats serotonin significantly decreased the period of LHRH pulses (54).

The superfusion technique is a useful in-vitro technique for examining the release of chemicals from a small piece of tissue. However, it is limited by the size of the tissue that can be used and the amount of time that the tissue remains viable. Fortunately the tissue (hypothalamus) that was investigated was small enough to be used with only minor manipulations (the individual hypothalami were bisected). The tissue viability is limited to about six hours of superfusion; therefore, all of the in-vitro experiments were limited to less than six hours of duration. The pulsatility of release could not be effectively determined during these relatively short periods of time. In-vivo experiments could be run for longer periods of time to determine the pulsatility of release.

IN-VIVO EFFECTS OF GABA STIMULATION:

All of the rats in the in-vivo studies were in the $OVXE_2$ hormonal state. Activation of hypothalamic GABAergic systems during this hormonal state decreased LHRH release in-vitro (figure 10). The push-pull perfusion technique has been successfully used by several investigators for in-vivo neuroendocrine studies (16,24,38,46,62,63,72). By measuring the chemical microenvironment in a specific location within the brain this technique allows the characterization of the systems that are interacting at that location. These characterizations can then be used (along with other data) to describe the relationships between components of a distinct neural system.

A behavioral evaluation was made in the push-pull perfusion experiments. The rats had their behavior evaluated hourly with a stimulus response test that was

quantitated with a response-reactivity scale. The rat was at all times maintained under conditions that minimized stress. No significant behavioral changes were seen during drug perfusion in any of these experiments. The response-reactivity rating scale was not discriminating enough to detect any changes in hypothalamic GABA activation. The stimuli used were too benign to detect a significant change in localized GABA activation. Using a more specific behavior, such as lordosis, was not possible due to the physical constraints of the push-pull perfusion apparatus. Also precluded were stimuli which elicited pain, because when reacting a rat could easily damage its apparatus. However, the innocuous stimuli used could not differentiate any change produced by activation of GABAergic systems within the median eminence. Only one of the previously cited neuroendocrine studies monitored the animal's behavior (38) and these animals were only observed to determine if mating had occurred. Attempting to quantify a behavioral change due to perfusion of a relatively small locus [one mm (46)] within the brain requires a very discriminating behavioral test.

None of the rats in the control experiments had any significant differences in the amount of LHRH released when the data was compared by collapsing ten minute intervals into hourly intervals. This was also true for the neurotransmitter data from these experiments. One rat was used as a vehicle control and no significant change was noted in the LHRH release throughout the experiment (figure 22). However, the 5-HIAA release did change significantly during the experiment (figure 23). The first hour and the last two hours of this experiment had significantly lower levels of 5-HIAA release. These hours included both control and vehicle perfusion and the significant difference does not reflect a change caused by vehicle perfusion. But rather the change reflects a normal fluctuation of 5-HIAA levels within the median eminence of this rat. The fluctuation in 5-HIAA release did not affect LHRH release since no significant change was seen in LHRH release during the entire vehicle control experiment.

Two of the five rats in the GABA experiments had significant differences in the amount of LHRH released during different hours of the experiment (figures 24 and 25). Unfortunately, the same hours were not different in each experiment. In the first experiment the first four hours of control perfusion were similar to the last two hours of GABA perfusion, while in the second experiment the first and last hour of control perfusion were different from the last two hours of GABA perfusion, as well as the third hour of control perfusion. However, in both experiments the last two hours of GABA perfusion had a significantly lower level of LHRH release than the first two hours of GABA stimulation. The cannula tip in both of these experiments was placed near the median eminence, with the tip placement in the first experiment being slightly rostral and medial to the position of the cannula tip in the second experiment. Two of the rats in the GABA experiments had significant differences in the amount of neurotransmitters released during different hours of the experiment. These were not the same rats that had differences in LHRH release. Most of these differences were seen between hours without release and hours with release (figure 26).

All six rats in the muscimol experiments did not have a significant difference in LHRH release during different hours of the experiment. Two of the rats in the muscimol experiments did have a significant difference in the amount of 5-HIAA released during different hours of the experiment. Unfortunately, the same hours are not different in these experiments. The cannula tips in these two experiments were located closer to the median eminence than any of the other rats in the muscimol experiments. The effect of muscimol on 5-HIAA release was different in these experiments. In one experiment muscimol seemed to increase 5-HIAA release, while in the other experiment muscimol decreased 5-HIAA release (figures 28 and 29). This could be due to differences in the diffusion of muscimol from the cannula tip. The muscimol may not have been able to decrease 5-HIAA release as quickly in one rat as it did in another simply because it took longer to reach the pertinent serotonergic

neurons. The experiment that showed an apparent increase in 5-HIAA release (figure 29) did have a significant decrease in 5-HIAA release during the last hour of muscimol perfusion. If the perfusion period had been longer this decrease may have continued.

The in-vivo experiments were very labor and cost intensive, and the total number of experiments was smaller. The increased between rat variability limited the data analysis to single rat comparisons. More than any other factor, the results of the in-vivo experiments depended on the placement of the cannula tip. An area of one mm surrounding the cannula tip was perfused in these experiments (46). The neurons that had axon terminals in that particular area were responsible for the chemicals present in the perfusate. As the cannula tip came closer to the median eminence, the amount of LHRH present in the sample was higher, as expected. These results cannot be extrapolated to generalize the relationships between the systems involved, but rather should be used to support other, more general, observations.

The in-vitro experiments provided the generalized observation that GABA can inhibit the release of LHRH in $OVXE_2$ rats. This was supported by results of two of the GABA perfusion experiments where decreases in LHRH release were seen. Unfortunately, because a live animal was being used in the in-vivo experiments a lower concentration of GABA (10^{-6}M versus 10^{-4}M) was necessary. This combined with the variability inherent in cannula placement made the in-vivo results more specific than the in-vitro results. However, by using the in-vivo results to corroborate the in-vitro results the specific aims of this research could be accomplished.

SUMMARY:

The effect of GABA stimulation on the hypothalamic reproductive neuroendocrine system appears to be inhibitory. This effect is dependent on plasma estradiol levels and changes throughout the rat estrus cycle. GABA does not regulate the frequency of pulsatile LHRH release but rather, the average amount of LHRH

released. Therefore, GABA seems to be exerting a modulatory effect on this system rather than directly affecting signal transmission. The role of GABA in the reproductive neuroendocrine system may be to synchronize the LHRH pulse generator.

Once the modulation of the hypothalamic reproductive neuroendocrine system by GABA is understood it can be exploited to aid in contraception and fertility treatments. This research determined that GABA can inhibit LHRH release under conditions of low, constant estradiol levels in the plasma. However, this inhibition is not mediated through the hypothalamic serotonergic system. GABA stimulation can affect the serotonergic system within the hypothalamus, but under conditions of rapidly increasing, high plasma estradiol levels. GABA may be inhibiting LHRH neurons during diestrus only to switch to inhibiting the serotonergic neurons or terminals during proestrus.

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